Identification of encoded beads

All patent and non-patent references cited in the present patent application are hereby incorporated in their entirety. This application is a non-provisional of U.S.
5   provisional application Serial No. 60/535,520 filed 12 January 2004, which is hereby incorporated by reference in its entirety.

Field of invention

10   The present invention relates to a spatially encoded polymer matrix in the form of a bead or a granule for combinatorial solid phase synthesis, assaying, functional proteomics, and diagnostic use. There is also provided a composition of such beads or granules. Each beaded polymer matrix of the composition comprises a plurality of spatially immobilised particles. The spatial immobilisation of the particles confers on
15   each beaded polymer matrix a "fingerprint" which enables identification of unique beads in a population of beads. The unique identification of individual beads makes it possible to perform combinatorial chemistry strategies while logging individual chemical transformation.

20   **Background of the Invention**

The synthesis of organic molecules on solid-phase synthesis beads has experienced an explosion of interest since Merrifield's pioneering work in the peptide area several decades ago. In large part, this renaissance has been driven by the advent
25   of combinatorial chemistry, which takes advantage of the ability to synthesize large and diverse libraries of compounds efficiently on solid support.

One inherent difficulty of producing large libraries by combinatorial chemistry is the problem of how to determine the reaction history in the form of the individual synthe-
30   sis steps resulting in the synthesis of any given combinatorial library member. Without such information it is not possible to deconvolute the structure of the combinatorial library member.

When employing a large number of solid supports and a large number of synthesis
35   steps and/or processing methods, the procedure of "deconvolution" is particularly

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difficult. In many practical cases, where high throughput screening and fast analysis is required, this problem is inherently associated with conventional methods for solid-phase synthesis.

5   Despite the tremendous practical advantages afforded by solid-phase synthesis, few reports have appeared in which a direct determination of the on-resin chemistry has been made possible in a practical way. Examples of techniques that have been used include radiography, nanoprobe nuclear magnetic resonance, single-bead fluo-rescence microscopy, IR spetroscopy, and optical analysis.

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Combinatorial libraries may be assembled by a number of methods including the "split-and-recombine" methods described e.g. by Furka et al. (1988, 14th Int. Congr. Biochem., Prague, Czechoslovakia 5:47; 1991, Int. J. Pept. Protein Res. 37: 487-493) and by Lam et al. (1991, Nature 354: 82-84), and reviewed by Eichler et al.

15   (1995, Medicinal Research Reviews 15 (6): 481-496) and by Balkenhohl et al. (1996, Angew. Chem. Int. Ed. Engl. 35: 2288-2337).

The split-and-recombine synthesis method involves dividing a plurality of solid sup-ports such as polymer beads into n equal fractions representative of the number of

20   available "building blocks" for each step of the synthesis (e.g., 20 L-amino acids, 4 different nucleotides etc.), coupling a single respective building block to each poly-mer bead of a corresponding fraction, and then thoroughly mixing the polymer beads of all the fractions together. This process is repeated for a total of x cycles to produce a stochastic collection of up to N^x different compounds.

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The conventional split synthesis technologies referred to above present difficulties when it is desired to detect and isolate a combinatorial library member of interest. In this regard, it is necessary to first cleave the member from its solid support before identifying the member by techniques such as mass spectroscopy or HPLC. This is

30   time consuming and cumbersome and in some cases, cleavage is not possible.

Janda (1994, Proc. Natl. Acad. Sci. USA 91: 10779-10785) describes a method in which each synthesis step of a combinatorial library member is followed by an inde-pendent coupling of an identifier tag to a solid support. Through a series of sequen-

35   tial chemical steps, a sequence of identifier tags are built up in parallel with the

compounds being synthesised on the solid support. When the combinatorial synthesis is complete, the sequence of operations any particular solid support has gone through may be retraced by separately analysing the tag sequence. Accordingly, use of identifier tags in this manner provides a means whereby one can identify the building blocks sequentially added to an individual solid support during the synthesis of a member of a combinatorial library.

WO 98/47838 discloses a method for the preparation of a chemical library on a plurality of synthesis particles comprising random features.

WO 93/06121 discloses a general stochastic method for synthesising a combinatorial compound library on solid supports from which library members may be cleaved to provide a soluble library. The identifier tag may be attached directly to a member of the library or to the solid support on which the member is synthesised. Tags such as oligonucleotides can be identified by sequencing or hybridisation. Amplification of oligonucleotide tags by PCR can be employed when only trace amounts of oligonucleotides are available for analysis. However, such identification methods are time consuming and inefficient.

US 5,721,099 discloses a process for constructing complex combinatorial chemical libraries of compounds wherein each compound is produced by a single reaction series and is bound to an individual solid support on which is bound a combination of four distinguishable identifiers which differ from one another. The combination provides a specific formula comprising a tag component capable of analysis and a linking component capable of being selectively cleaved to release the tag component. Prior to analysis of a combinatorial library, each tag component must be cleaved from the support thus creating at least one additional step which is time consuming and inefficient.

Also, the above methods all rely on parallel, orthogonal synthesis of identifier tags which adds substantially to the time taken for completion of a combinatorial synthesis and has the potential of interfering with the synthesis.

Spectrometric encoding methods have also been described in which decoding of a library member is permitted by placing a solid support directly into a spectrometer

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for analysis. This eliminates the need for a chemical cleavage step. For example, Geysen et al. (1996, Chem. Biol. 3: 679-688) describe a method in which isotopically varied tags are used to encode a reaction history. A mass spectrometer is used to decode the reaction history by measuring the ratiometric signal afforded by the

5   multiply isotopically labelled tags. A disadvantage of this method is the relatively small number of multiply isotopically labeled reagents that are commercially available.

Optical encoding techniques have also been described in which the absorption or

10   fluorescence emission spectrum of a solid support is measured. Sebestyén et al. (1993, Pept. 1992 Proc. 22nd Eur. Pept. Symp. 63-64), Campian et al. (1994, In Innovation and Perspectives on Solid Phase Synthesis; Epton, R., Birmingham: Mayflower, 469-472), and Egner et al. (1997, Chem. Commun. 735-736) have described the use of both chromophoric and/or fluorescent tags for bead labeling in

15   peptide combinatorial synthesis. Although this use provides an advantage for deconvoluting the structure of a library member by determining the absorption or fluorescence emission spectrum of a bead, the encoding of a large library would require the use of many chromophores or fluorophores where spectral superimposition would be a likely drawback.

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WO 95/32425 discloses the coupling on beads of (i) fluorescently labelled tags having intensities that differ by a factor of at least 2, and/or (ii) multiple different fluorescent tags that can be used in varying ratios, to encode a combinatorial library. Such beads may be used in concert with flow cytometry to construct a series of combina-

25   torial libraries by split synthesis procedure. Although this method has advantages in relation to providing a lead structure, it is necessary to construct and analyse multiple libraries commensurate with the number of stages used for the combinatorial synthesis, which is cumbersome and time consuming.

30   WO 97/15390 describes a physical encoding system in which chemically inert solid particles are each labelled with a unique machine readable code. The code may be a binary code although higher codes and alphanumerics are contemplated. The code may consist of surface deformations including pits, holes, hollows, grooves or notches or any combination of these. Such deformations are applied by micro-

35   machining. Alternatively, the code may reside in the shape of the particle itself. Solid

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particles comprising a first phase for combinatorial synthesis and a second phase containing a machine readable code are exemplified wherein the second phase may be superimposed on, or encapsulated within, the first phase. The microscopic code on the particles may be interrogated and read using a microscope-based image cap-
5   ture and processing system. The machine readable code may be read "on-line" between different process steps of a combinatorial synthesis thus allowing the process sequence, or audit trail, for each bead to be recorded.

     Nano bar coding for bioanalysis has also been described by Keating, Natan and co-
10   workers (Science, 2001, vol. 294, 137).

     Xu et al. (2003) Nucleic Acid Research 31(8):e43 describes the use of combinations of fluorescent semiconductor nanocrystals to encode microspheres. The nanocrystals are too small to allow visualisation of their spatial location in the bead. Further-
15   more, as overlap of emission spectra needs to be avoided, the number of different nanocrystals that can be used in one bead will be limited.

     WO 00/32542 discloses high throughput screening based on carriers having distinctive codes such as electronmagnetic radiation-related compounds. Similar methods
20   have been described by Battersby et al. (2001, Drug Discovery Today, vol. 6, no. 12 (Suppl.), S19-26); Battersby and Trau (2002, Trends in Biotechnology, vol. 20, no. 4, 167-173; Meza (2000, Drug Discovery Today, vo. 1, no. 1, 38-41), and by Farrer et al. (2002, J. Am. Chem. Soc., vol. 124, no. 9, p.1994-2003).

25   Many of the disadvantages of the known methods described above, as well as many of the needs not met by these methods, are overcome by the present invention, which, as described herein below, provides several advantages over the above-described prior art methods.

30   **Summary of the Invention**

     It is a first object of the present invention to prepare libraries of specific active compounds with respect to biochemical interactions, such as substrate catalysis, purification or isolation of desirable targets, as well as studying host-guest interactions,
35   and preferable materials properties.

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This is exemplified by compounds such as e.g. polypeptides (alpha-peptides, beta-peptides, and the like), polynucleotides (DNA, RNA, LNA and PNA, including non-natural and modified nucleotides comprising non-natural or modified nucleobases and/or backbones and/or carbohydrate moieties), as well as carbohydrates, scaffolded small molecules, and mimetics of these compound classes. The libraries are preferably bead based and offer as such several advantages over prior art libraries based on chips and similar solid supports.

The active compounds of the invention can be screened for the identification of novel ligands that interact with e.g. a receptor target of interest. As such, the active compounds can be used e.g. for identifying or further develop potential drug candidates, new catalysts and materials with novel functionalities. One important application of the libraries is in the diagnostic and functional proteomics area. In the following the use is exemplified by the screening of biological targets.

For any given receptor target, the probability of successfully identifying a potent ligand through a process of randomly screening molecular repertoires will increase as the size and structural diversity of the library is also increased. The present invention makes it possible to i) rapidly identify an individual bead in a composition of beads based on individual bead "fingerprints", and ii) immediately "deconvoluting" the sequential steps employed in the solid-phase synthesis of the biologically active compound on the individual bead in question.

In order to solve this problem the invention provides in a first aspect a beaded polymer matrix in which particles, also termed "microbeads" herein, have been immobilized in a random spatial arrangement such that each bead can preferably be uniquely identified by the 3-dimensional pattern formed by the particles. The particles can be labelled particles made from the same polymer material as the base synthesis polymer or they can be composed of a different material. The pattern can be detected by a property of the particle that differs from the surrounding polymer matrix. This difference can be achieved by fluorescence labels, colour labels or by different diffractive or reflective properties of the immobilized material.

The uniquely labelled particles can be divided into portions with recording of their identity and location and subjected to different reaction conditions accordingly. Using combinatorial methods such as "Split and recombine" synthesis it is thus possible to record the precise history of reactions for each particle and thereby the structure of the product formed for each unique bead.

It is furthermore possible after screening and isolation of active hits to identify the structure of the active beads by recording the encoding pattern of the bead and correlate the pattern with all patterns recorded during the synthesis.

It is also possible to use the tool to perform diagnostic tests with mixtures of active ligands on encoded beads and after measuring the clinical values from the beads decode the results by reading the pattern of the beads.

Additionally, the invention makes it possible to purify and/or isolate targets from a mixture potentially comprising a target having an affinity for a ligand attached to a beaded polymer matrix. The isolation of the target can involve a chromatographic separation step resulting in the separation of the target from the additional components of the mixture. Affinity chromatography as well as any other chromatographic separation step can be employed for the separation and/or isolation of target compounds from a mixture of compounds.

In one aspect the invention provides an encoded beaded or granulated polymer matrix, preferably a matrix suitable for solid phase synthesis or chromatographical applications, said matrix comprising a plurality of spatially immobilised particles or vacuoles, wherein each particle or vacuole is individually detectable.

In another aspect the invention provides a composition comprising a plurality of different, spatially encoded, wherein essentially each bead is individually identifiable.

In order to detect individual beads there is provided in another aspect a method for the detection of relative positions in space of centers (x,y,z) of immobilized particles of the composition according to the invention described herein above, said method comprising the step of recording of at least two 2D-projections of the particles, said method optionally comprising the further step of determining, on the basis of the

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relative positions in space of centers (x,y,z) of immobilized particles, the distance matrix for individual beads, or a set of geometrical figures, preferably selected from triangles and quadrangels, more preferably triangles, wherein said geometrical figures are derivable from either the above-mentioned distance matrix, or from the relative positions in space of centers (x,y,z) of the immobilized particles.

For determination of the distance matrix of a beaded or granulated polymer matrix according to the invention there is provided in accordance with the present invention a method for distance matrix determination of at least one spatially encoded beaded or granulated matrix comprising a plurality of spatially immobilised particles comprising an optically detectable label, said method comprising the steps of

i) providing at least one beaded or granulated polymer matrix according to the invention,

ii) providing at least one device for recording and storing at least one image of the at least one bead, said device comprising

a) at least one source of illumination,
b) at least one system for manipulating and optionally for sorting beaded or granulated polymer matrices comprising an imaging section, such as e.g. a flow system comprising a flow cell comprising an imaging section
c) at least one image capturing device, such as e.g. a CCD camera,
d) optionally at least one pulse generator, and
e) further optionally at least one image intensifier,

iii) activating the at least one source of illumination,

iv) introducing the at least one encoded bead comprising a plurality of particles into the imaging section of the system,

v) recording at least one image of the at least one bead, preferably by sending substantially simultaneously a pulse generated by a pulse generator to both a) the at least one image intensifier, and b) the at least one

image capturing device, such as a CCD camera capable of recording said at least one image, and

vi)   determining for at least one individual bead a distance matrix based on the at least one image obtained in step v).

In another aspect there is provided a method for identifying individual beaded polymer matrices in a composition according to the invention, said method comprising the steps of

i)   determining the distance matrix for individual beads of a bead population according to any of the methods of the invention,

ii)   deriving from each of the distance matrices generated in step i) all of the geometrical figures, such as triangles, which can be generated by connecting particle coordinates with straight lines, and

iii)   recording and storing the set of geometrical figures for substantially each and all of the beads of the population to be identified,

iv)   optionally performing a method involving a solid phase synthesis step for synthesising at least one compound and/or a step for purifying and/or isolating at least one binding partner having an affinity for said compound,

v)   selecting a subset of beads, and

vi)   identifying one or more of the selected beads on the basis of a comparison of the set of geometrical figures of said bead(s) with all sets of geometrical figures recorded for substantially each and all of the beads of the population in step iii).

There is also provided in another aspect a method for identifying at least one individually identifiable, spatially encoded, bead in a composition according to the invention, said method comprising the steps of

i)   determining the unique, spatial position of three or more particles in the at least one bead to be identified,

ii)   deriving from the positions, a matrix of the distances between the three or more particles,

iii)   deriving from the matrix, a set of all geometrical figures, such as trian-
         gles defined by the coordinates of three or more particles,

iv)   identifying said at least one individually identifiable, spatially encoded
         bead based on comparison of the set of triangles for the bead in ques-

5   tion with all sets of triangles for essentially all beads of the composition
         of the invention comprising different spatially encoded beads.

When, in any of the above methods, the identification of an individual bead is per-
formed by comparing a set of geometricla figures, such as triangles, for the bead to

10   be identified, with the set of geometrical figures, such as triangles, for all beads of
the population of beads, the method for identifying an individual bead, b_q, of a plu-
rality of beads, $B = (b_1, b_2, ..., b_H)$, where $1 \leq q \leq H$, and H being the number of
beads, H preferably being less than 10^{17}, such as less than 10^{15}, such as less than
10^{13}, such as less than 10^{11}, such as less than 10^{10}, for example less than 10^9, such

15   as less than 10^8, for example in the range of from 10^3 to 10^7, preferably comprises
the steps of

providing a plurality of spatially encoded beads, B,

20   obtaining at least one orthogonal pair of images, $(I_{h,x,z}, I_{h,y,z})$, of each bead, b_h,
where $h = 1, 2, ..., H$, of said plurality of distance encoded beads, B,

deriving from each of said at least one orthogonal pair of images, $(I_{h,x,z}, I_{h,y,z})$, the
set, C_h, of possible sets of three-dimensional particle positions represented by x, y,

25   and z image pixel values for each bead, b_h,

$C_h = (c_{h,1}, c_{h,2}, ..., c_{h,Eh})$, where $c_{h,e} = (x_{h,f,e}, y_{h,f,e}, z_{h,f,e})$,

where $f = 1, 2, ..., F_h$, and F_h being the number of particles of bead b_h, and e

30   $= 1, 2, ..., E_h$, and E_h being the number of possible sets of three-dimensional
particle positions for bead b_h,

deriving for each set of possible sets of three-dimensional particle positions one
distance matrix

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$$D_{h,e} = \begin{vmatrix} 0 & d_{h,e,1,2} & d_{h,e,1,3} & \dots & d_{h,e,1,Fh} \\ d_{h,e,2,1} & 0 & d_{h,e,2,3} & \dots & d_{h,e,2,Fh} \\ d_{h,e,3,1} & d_{h,e,3,2} & 0 & & \dots \\ \dots & \dots & & \dots & \\ d_{h,e,Fh,1} & d_{h,e,Fh,2} & \dots & & 0 \end{vmatrix}$$

where $d_{h,e,i,j}$ = integer($[(x_{h,e,i}-x_{h,e,j})^2+(y_{h,e,i}-y_{h,e,j})^2+(z_{h,e,i}-z_{h,e,j})2]^{\frac{1}{2}}$), where i = 1,2,...$F_h$, and j = 1,2,...,F_h ,

deriving for each distance matrix, $D_{h,e}$, the full set of derivable triangles, $T_{h,e}$ = ($t_{h,e,1}$, $t_{h,e,2}$, ..., $t_{h,e,Ghe}$), each triangle being represented by its three side length,

$T_{h,e}$ = $[t_{h,e,1}, t_{h,e,2},..., t_{h,e,Ghe}]$ = $[(d_{h,1,2}, d_{h,1,3}, d_{h,2,3}), (d_{h,1,2}, d_{h,1,4}, d_{h,2,4}),...,(d_{h,(Fh-2),(Fh-1)}, d_{h,(Fh-2),Fh}, d_{h,(Fh-1),Fh})]$,

$G_{h,e}$ being the total number of derivable triangles from distance matrix, $D_{h,e}$,

generating a subset, U, of all triangles, T, derived for the full set of beads, B, said subset of triangles comprising all different triangles derived for the full set of beads,

U = (u_1, u_2, ..., u_W),

where $u_i \neq u_j$, for $i \neq j$, and i = 1, 2, ..., W, and j = 1, 2, ..., W, and W being the total number of different triangles derived for the full set of beads, B,

generating a look-up table, L, that for every triangle, u_r , where r = 1, 2, ..., W, gives the subset, A_r, of the full set of beads, B, for which subset of the full set of beads at least one of its derived sets of triangles comprises u_r,

L = $[(u_1, A_1), (u_2, A_2), ..., (u_W, A_W)]$,

obtaining at least one orthogonal pair of images ($I_{q,x,z}$, $I_{q,y,z}$) of the bead, b_q, to be identified,

deriving from said at least one orthogonal pair of images ($I_{q,x,z}$, $I_{q,y,z}$) the full set of possible sets of three-dimensional particle positions,

$$C_q = (c_{q,1}, c_{q,2}, \dots c_{q,Eq}),$$

deriving for each of said sets of possible sets of three-dimensional particle positions one distance matrix

$$D_{q,e} = \begin{vmatrix} 0 & d_{q,e,1,2} & d_{q,e,1,3} & \dots & d_{q,e,1,Fq} \\ d_{q,e,2,1} & 0 & d_{q,e,2,3} & \dots & d_{q,e,2,Fq} \\ d_{q,e,3,1} & d_{q,e,3,2} & 0 & & \dots \\ \dots & \dots & & \dots & \\ d_{q,e,Fq,1} & d_{q,e,Fq,2} & \dots & & 0 \end{vmatrix}$$

deriving for each distance matrix, $D_{q,e}$, the full set of derivable triangles, $T_q = (t_{q,e,1}, t_{q,e,2}, \dots, t_{q,e,Gqe})$, each triangle being represented by its three side length,

$$T_q = [t_{q,e,1}, t_{q,e,2}, \dots, t_{q,e,Gqe}] = [(d_{q,1,2}, d_{q,1,3}, d_{q,2,3}), (d_{q,1,2}, d_{q,1,4}, d_{q,2,4}), \dots, (d_{q,(F-2),(F-1)}, d_{q,(F-2),F}, d_{q,(F-1),F})],$$

finding for each of said triangles of said set of triangles, T_q, derivable from bead b_q the corresponding set, B_q, of subsets of beads according to said look-up table, L, for which at least one of its derived sets of triangles comprises each of said triangles of said set of triangles, T_q, derivable from bead b_q,

registering for each of the beads of said subset of beads, B_q, the number of triangles contained in T_q,

identifying bead b_q as the bead of said subset of beads, B_q, that has the highest number of triangles contained in T_q.

When the phrase "all possible sets of particle positions" is being used herein above, it will be understood that based on at least one 2D projection, more than one 3D interpretation may exist due to correspondance problems as described elsewhere herein.

Therefore, in one preferred embodiment of the present invention, "all possible sets of particle positions" shall mean "essentially all" possible set of particles positions, such as in more than about 90%, such as in more than 95% of all cases, "all possible sets of particle positions" shall mean "all sets of particle positions" for the bead in question.

The problem is that it may not always be possible to determine for each and every bead, the total number of geometrical figures for individual beads. As an example, when one particle is interfering with the imaging of another particle, e.g. by shielding another particle, it may not be possible to determine for such a shielded particle all coordinates x,y,z for a given microparticle.

In a further aspect of the invention there is provided a method for recording individual reaction steps involved in the step-wise synthesis of a chemical compound on a beaded polymer matrix according to the invention, said method comprising the steps of

a) spatially immobilizing a plurality of particles in polymer beads or granulates,

b) isolating, preferably by automated selection, at least a subset of the spatially encoded beads or granulates provided in step a), and

c) recording and storing a distance matrix or a geometrical figure derivable from the distance matrix for each bead or granule, said distance matrix or geometrical figurde being preferably generated by the methods of the invention disclosed herein,

d) stepwise synthesising chemical compounds on functional groups of the encoded beads or granules, wherein the identy of each bead or granule is recorded and stored for each reaction step, and

e) obtaining for each bead a record of individual reaction steps.

In conjunction with the above method for recording individual reaction steps involved in the step-wise synthesis of a chemical compound on a beaded polymer matrix according to the invention there is provided a method for identifying a chemical

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compound being synthesised on a beaded polymer matrix according to the invention, said method comprising the steps of

a) performing the recording method cited herein immediately above,

b) selecting beaded polymer matrices or granules of interest by using an assay or a diagnostic screen selective for the chemical compound having been synthesised on the beaded polymer matrix,

c) recording the distance matrix for each of the beaded polymer matrices selected in step b),

e) comparing the distance matrix recorded in step c) with all of the distance matrices recorded and stored in step c) of the above method, thereby obtaining information about the identity of the selected bead,

f) identifying for each selected bead the sequence of individual steps having lead to the synthesis of the chemical compound, and

g) identifying, based the sequence of individual steps the chemical structure of the compound.

For performing the above methods there is provided a device for recording and storing at least one image of at least one spatially encoded bead comprising a plurality of particles, said device comprising

i)   at least one source of illumination,

ii)   a flow cell comprising an imaging section,

iii)   at least one pulse generator,

iv)   at least one image intensifier, and

v)   at least one CCD camera.

There is also provided a method for generating a beaded or granulated polymer matrix comprising a plurality of spatially immobilised particles according to the invention, said method comprising the steps of

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> a) synthesizing a monomer or macromonomer and a crosslinker for po-
> lymerization, and

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> b) mixing these with the encoding particles to give an even dispersion of
> particles in the mixture, and

polymerizing the monomer or macromonomer by either i) suspension polymerisation
and/or; ii) inverse suspension polymerisation and/or iii) bulk polymerisation followed
10 by granulation, and/or iv) droplet polymerisation.

Brief Description of the Drawings

15 Scheme 1: The immobilisation of fluorescently labelled small PEGA-particles in a
PEGA-polymer by inverse suspension polymerisation of a mixture of small labelled
particles with partially acryloylated bis-amino PEG

Scheme 2: Polymers particularly useful for immobilization of encoding particles are:
20 A) PS, B) POEPS, C) POEPOP, D) SPOCC, E) PEGA, F) CLEAR, G) Expansin, H)
Polyamide, I) Jandagel or derivatives of any of these. Alginates, gelatines, aluminas,
pore glasses and silicas are other types of useful supports.

Figure 1: The principle of recording of coordinates for encoding particles in a bead
25 and conversion to a orientation independent distance matrix that uniquely identifies
the single bead. Three unique distances within 500 micron beads comprising 1 mi-
cron particles there is 65.449.846 positions giving in theory $\sim 3 \times 10^{23}$ combinations
from which at least 3×10^{15} will be unique. Orthogonal recording on three LCD de-
tectors yields 9 co-ordinates. These can be paired based either on fluorescence
30 intensity or on a fourth non orthogonal detector. Conversion to inter-particle dis-
tances gives an orientation independent parameter set. The 3 distances (3 particles)
are sorted and indexed according to the longest distance. Beads are sorted accord-
ing to number (3, 4 or 5) of particles prior to use. If beads with 4 particles are used,
6 distances are stored etc. Alternatively, two scanning lasers can directly yield the
35 three coordinate sets on the moving bead.

Figure 2. Recording of particle coordinates with 3 ccd's for xy-plane placed along 3 orthogonal axis by excitation of the bead with a single laser pulse.

5   Figure 3. Recording of coordinates of particles in a bead by focal or confocal microscopy.

Figure 4. Recording of coordinates of particles in a moving bead by two alternating scanning lasers.

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Figure 5. Recording of coordinates of particles using a single laser and a rotating mirror with reflection from 3 angles.

Scheme 3. Split and combine synthesis of a library of 400-dipeptides on 1000 beads

15   with reading of encoding at each reaction step.

Figure 6 A-I. Selected examples of pictures of 20 selected beads shown with the pool of identification out of 20 possible for each reaction step.

20   Figure 7 A and B. Two sets of 3 orthogonal pictures with 3 fluorescent particles immobilised in a bead.

Figure 8. Monte Carlo simulation of 10'000 beads of 500 units diameter. Using 4 fluorescent particles, the relative frequencies for different encoding separations $\cap$v

25   (see text) are plotted. The main figure is given on the basis of 100'000 out of the

total of $\binom{10^4}{2}$ Z 50 million pair-wise distances between the encoding vectors, while

the inset uses all 50 millions. Note that only in 2 out of 10'000 cases $\cap$v P 12 units, and that in none of the 50 million pairs $\cap$v P 3 units.

30   Table 1. Visually identified beads in each reaction step indicating amino acid sequence and verification of the result by Edman degradation sequencing.

Fig 9. Illustration of the correspondence problem in the case where a pair of orthogonal fluorescence images (right) give rise to two spatial interpretations (left).

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Fig. 10. Illustration of the correspondence problem in the case where a pair of orthogonal fluorescence images (right) give rise to each one 3D-interpretation (left) due to the difference in sharpness and intensity of individual spatially immobilised particles.

Fig. 11. C versus M and δ (upper), E versus M and δ (lower). δ is given in micrometers.

Fig.12. One embodiment of the encoded bead reader.

Figure 13. Micrographs of micro beads before (upper) and after (lower) centrifugation. 20X-lens was used. The black lines in the image are 50 micrometers long.

Figure 14. Size distribution before and after centrifugation. (Total number of micro beads measured was 93 before centrifugation and 88 after centrifugation.

Fig. 15. Schematics of tracing hits backwards through a combinatorial chemistry route. This requires that all encoded beads are read by an encoded bead reader when fed to a reaction jar (small cups in the image) and/or when removed from a reaction jar during the combinatorial chemistry synthesis.

Fig. 16. Fluorescence images of stained microbeads JHT466 (upper left), JHT472 upper right, JHT471 (lower left), and JHT473 (lower right). The black bars in the images are 25 micrometers long.

Fig. 17. Encoded bead from sample JHT476. The black lines in the image are 80mm long.

Fig. 18. A pair of orthogonal fluorescence images of an encoded bead from batch JHT483. The upper image is the x,z-projection, with the x-direction upwards and the z-direction to the right. The lower image is the y,z-projection, with the y-direction upwards and the z-direction to the right. The images measure 1mm x 1mm.

Fig. 19. The number of encoded beads with correspondence problem, C, versus the average number of immobilised particles per encoded bead, M and the uncertainty involved in the determination of the immobilised particle positions, δ (upper), and the number of not identified encoded beads, E, versus M and δ (lower). δ is given in micrometers.

Fig. 20. Illustration of two pairs of orthogonal images based on experiment disclosed in Example 18.

Fig. 21. Orthogonal fluorescence image pairs of three spatially encoded beads. The images measure about 1.2mm x 1mm.

Definitions

Beaded polymer matrix: A beaded polymer matrix is a crosslinked polymer formed by beading according to principles of suspension or inverse suspension polymerization, by spray polymerization, or by droplet polymerisation.

Bioactive compound: Molecules comprising a sequence of building blocks, which includes e.g. L-amino acids, D-amino acids, or synthetic amino acids, such a beta-amino acids, as well as natural and non-natural nucleotides and polynucleotides, and carbohydrates. It will also be understood that different basis sets of building blocks may be used at successive steps in the synthesis of a compound of the invention.

Carrier: Used interchangably with a beaded polymer matrix or a granulated polymer matrix.

Code: Used interchangably with the unique nature of individually identifiable beads or granules the identification of which resides in the unique spatial distribution of a plurality of particles or vacuoles. The code for each bead or granule is unique.

Coordinates: The coordinates are relative spatial coordinates assigned to particles in the bead

2 D-coordinates: these are coordinates of particles in a 2-D projection of the particle along one of three orthogonal axes.

Encoded beaded polymer matrix: This is a beaded polymer matrix formed by polymerization of a monomer mixture comprising a dispersion of particles.

Essentially: This term signifies that a physical process often yields a result that deviates from the theoretical result expected due to inheterogeniety and incomplete control of the process.

Essentially monodisperse: This indicate that a slight tendency towards inhomogenous location of particles can be expected due to differences in density and aggregation phenomena.

Essentially spherical: Any spherical object for which the distance from the gravitational centre to any point on the surface of the object is in the range of from a quarter of the average distance from the gravitational centre to the surface to preferably less than four times the average distance from the gravitational centre to the surface.

Essentially the same diameter: The diameters are never identical since a gaussian distribution of bead sizes is obtained during polymerization

Fluorescently detectable: An unsaturated organic molecule, a complex, an alloy or a transition metal that is excited at one wavelength and due to electronic structure and heat emmision return to ground state with emmision of a photon at a different wavelength, which can be detected.

Granule: An essentially spherical object having an irregular form.

HYDRA: PEG-triaminoethylamine star copolymer.

Individually detectable: This refer to the separation of beads in a fluidic stream of beads that allow recording of the encoding pattern of each individual bead.

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PEGA: PEG-acrylamide copolymer (may be alkylated on amide)

Photon fluorescence spectroscopy: One photon fluorescence spectroscopy, which is the same as standard fluorescence spectroscopy, is based on the facts that

5   a molecule can be excited by a single photon, and that the excited molecule after a internal process emits a photon with a lower energy than the excitation photon. The energy (the spectrum) as well as the rate of emission is specific for the molecule in its specific environment. Two-photon excitation of fluorescence is based on the principle that two photons of longer wavelength light are simultaneously absorbed by a

10   fluorochrome which would normally be excited by a single photon, with a shorter wavelength. The non-linear optical absorption property of two-photon excitation limits the fluorochrome excitation to the point of focus.

POEPOP: Polyethyleneglycol-polyoxypropylene copolymer

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Resolution: This term refers to the resolution of a detection method, in a ccd frame-grap this is defined by the number of pixels and the optics used to produce the picture, in a scanning laser detection this relates to the cross-section of a laser beam at the point of excitation.

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Solid phase synthesis: Synthesis where one of several of the reactants forming the target molecule is attached to a solid support e. g. a beaded polymer matrix.

Spatial position: Position of a bead or particle in space defined by Cartesian coordi-

25   nates

Spatially immobilised particles: Particles which are immobilized in a surrounding polymer matrix in such a way that the individual distances between the immobilized particles are constant in a particular solvent.

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SPOCC: Polymer obtained by ring opening polymerisation of partially or fully 3-methyloxetan-3-ylmethyl alkylated PEG.

Swelling: When beads or granules or particles or vacuoles are capable of swelling,

35   any physical measurement of the afore-mentioned, including size determinations

and volume determinations, refer to measurements conducted for the swelled bead or granule or particle or vacuole. Swelling of the beads are for practical reasons measured as the volume of a packed bed of beads swollen in a specific solvent and divided by the dry weight of the beads. The unit is given as ml/g. Typical solvents are water, methanol and dichloromethane, but any suitable solvent may be chosen. When the refractive index of the swollen bead or granule is different from the refractive index of the surrounding solvent the swollen bead or granule will function as an optical lens. When the relative positions of immobilised particles inside the swollen beads or granules are determined by optical means this lens effect may give rise to inaccurate determination of the relative positions of the immobilised particles. Preferred solvents give rise to as little difference in refractive index between the solvent and the swollen bead or granule as possible. For instance when the polymer matrix comprises cross-linked polyethyleneglycol a one-to-one mixture of ethanol and glycerol gives rise to nearly no refractive index difference.

Unique distance matrix: Each bead is uniquely identified by an orientation independent distance matrix describing the relative positions of particles within the encoded bead.

Uniquely identifiable: Used herein interchangeably with "individually identifiable", i.e. that a single bead can be identified on the basis of the spatial configuration of the particles immobilised in the bead. The encoded beads are "individually identifiable" within the limits of statistical probability of occurrence of identical beads and resolution of identification method. In one embodiment, with a practical resolution of 1:100 and only 4 encoding particles the probability of e. g. selecting two identical beads is 10^{-6} according to Monte-Carlo simulation. A total of $\sim 10^{15}$ different beads may be encoded. More preferably, more than 95%, such as more than 97%, for example about or more than 98%, such as about or more than 99% of all beads will be "individually identifiable" under practical circumstances.

Vacuole: Space comprising gaseous or liquid composition of matter, wherein said matter is identifiable by having at least one spectroscopically or optically detectable parameter which distinguishes the vacuole from the beaded polymer matrix.

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Detailed Description of the Invention

Distance matrix determination

5   In one embodiment, the spatial immobilisation of the plurality of particles in each beaded polymer matrix is essentially unique for each bead. The spatial positions of particles in each bead can be defined by sets of coordinates, (x,y,z) of particle centers of said particles, relative to one reference point of the detection. Furthermore, the relative positions in space of centers (x,y,z) of immobilized particles can be detected based on recording of 2D-projections of the particles.

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In one embodiment, 3 2D-projections are recorded along 3 orthogonal axis x, y and z to generate 3 sets of 2D-coordinates (y,z), (x,z) and (x,y), respectively, from which the 3D-coordinates (x,y,z) of particle centers can be derived. A stack of 2D projections can be generated by confocal or focal microscopy to recreate the 3D image matrix of the bead from which the relative particle position (x,y,z) in space can be determined.

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One method for determination of relative particle positions within a bead can be based on focussed scanning lasers and laminar fluidics, preferably methods in which the coordinates x and y of a particle position is determined by fast scanning two orthogonally aligned lasers over two cross sections of the moving bead while the z coordinate is determined by the time of flight of the bead at known flow rates. Accordingly, it is possible to determine the coordinates x and y of a particle position by using a single laser and a rotating mirror that via 2 or three geometrically arranged static mirrors reflects the laser beam along 2 or 3 orthogonal axis

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Accordingly, one method for recording the unique pattern of each encoded bead comprises the steps of recording the relative coordinates of the center of the spatially immobilised particles and calculating a distance matrix based on the recorded coordinates. Accordingly, it is possible to convert the relative coordinates into absolute and unique parameters for each bead by generating for each bead a distance matrix of inter particle distances.

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The coordinates of the particles in a bead can be generated in a variety of different ways.

1. A laser or conventional light excitation of the entire bead can be combined with detection along 3 orthogonal axis with three CCD cameras and the three sets of coordinates measured in 2D X,Y; Y,Z and X,Z for each particle can be used to correlate the particles to give a unique set of parameters XYZ for each immobilized particle.

2. A principle of focal or confocal microscopy can be used to obtain a 3D representation of the bead in which the 3 coordinates are the x and y of the particle in the a particular picture while the z-coordinate is derived from the focal depth.

3. Using fluorescence labelled particles a set of two focussed alternating scanning lasers along two orthogonal axis can excite the fluorophores on a moving particle in a flowcell and the fluorescence recorded with a pmt. The coordinates are generated from the two excitation positions and the position of the bead in the fluidic stream. This bead position is measured by the time of flight of the bead as determined from extinction measurement on one of the lasers.

The methods and spatially encoded beads described above can be used to identify single beads out of a very large assembly of beads by rapid decoding at any point of process time. They can furthermore be used in connection with diagnostic kits where a large mixture of beads are used in a fashion similar to that of spatial arrays of e. g. DNA or protease substrates.

When polymer beads encoded with spatially immobilised particles are to be identified by the distance matrix between said spatially immobilised particles, the relative position of each particle must be determined unambiguously within some acceptable experimental error.

To a large extent this can be done by multiple imaging or laser scanning. However, erroneous distance matrices may result in cases where the optical data obtained

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gives rise to two or more three-dimensional (3D) interpretations. For instance, when encoded beads are viewed from two orthogonal angles corresponding to an x,z-projection and a y,z-projection, a "correspondance problem" arises when two or more spatially immobilised particles have the same z-value within the optical accuracy of the equipment. One example of the "correspondance problem" arising from one set of images giving rise to two or more possible 3D-structures is illustrated in Fig. 9.

Below is provided three non-limiting examples of conceivable solutions to the "correspondence problem" illustrated in Fig. 9:

Solution 1: Focal depth evaluation

Spatially immobilised particles positioned at the focal plane of the imaging objective appear as sharp and intense bright spots, whereas particles positioned away from the focal plane of the objective appear as less sharp and less intense, the sharpness and intensity gradually decreasing as the distance from the particle to the focal plane increases.

In case that the dimensions of the imaging section exceeds the focal depth of the objectives, any 2D-projection will - apart from giving the 2D-positions of each particle - also provide information about the distance of each microbead from the focal plane. This information can be used to distinguish between spatially immobilised particles which are otherwise indistinguishable or result in the calculation of more than one distance matrix.

One example of the correspondence problem is given in figure 10.

Solution 2: Principal component projection

This solution is provided essentially by performing the method steps listed herein below:

    1. Obtaining an orthogonal pair of images of each spatially encoded bead,

2. Determining the 2D-positions of each spatially immobilised particle in each of said two orthogonal images,

3. Combining the resulting two orthogonal sets of 2D-positions whereby the set of possible sets of 3D-positions is obtained for each spatially encoded bead.

4. Calculating the principal component axis, x', y', z', of one of set of possible sets of 3D spatially immobilised particle positions.

5. Calculating the projected set of 3D spatially immobilised particle positions by projecting the 3D spatially immobilised particle positions onto said principal component axis.

6. Calculating the projected distance matrix based on the projected set of 3D spatially immobilised particle positions.

7. Identifying single spatially encoded beads by comparing the full set of projected distance matrices of single spatially encoded beads against the full set of projected distance matrices of all spatially encoded beads. The best fit of single projected distance matrices hereby obtained identifies single spatially encoded beads.

Encoded bead identification based on the principal component projected distance matrix is considerably more stable towards mismatching of spatially immobilised particles than encoded bead identification based on the conventional distance matrix.

Solution 3: Multiple distance matrix calculation

A multiple distance matrix can be calculated by performing the steps of the following method:

1. Obtaining two orthogonal pairs of images of each spatially encoded bead,

2. Determining the 2D-positions of each spatially immobilised particle in each of said two orthogonal images,

3. Combining the resulting two orthogonal sets of 2D-positions, whereby a set of 3D-positions is obtained for substantially each spatially encoded bead,

4. Computing the set of distance matrices corresponding to the set of 3D-positions thus determined in step 3,

8. Identifying single spatially encoded beads by comparing the full set of distance matrices of single spatially encoded beads against the full set of sets of distance matrices of all spatially encoded beads, wherein the best fit of single distance matrices hereby obtained identifies single spatially encoded beads.

In one preferred embodiment of the method for calculating multiple distance matrices, the identification of an individual bead is performed by comparing a set of geometricla figures, such as triangles, for the bead to be identified, with the set of geometrical figures, such as triangles, for all beads of the population of beads, the method for identifying an individual bead, b_q, of a plurality of beads, $B = (b_1, b_2, ..., b_H)$, where $1 \le q \le H$, and H being the number of beads, H preferably being less than 10^{17}, such as less than 10^{15}, such as less than 10^{13}, such as less than 10^{11}, such as less than 10^{10}, for example less than 10^9, such as less than 10^8, for example in the range of from 10^3 to 10^7, preferably comprises the steps of

1. providing a plurality of spatially encoded beads, B,

2. obtaining at least one orthogonal pair of images, $(I_{h,x,z}, I_{h,y,z})$, of each bead, b_h, where h = 1, 2, ..., H, of said plurality of distance encoded beads, B,

3. deriving from each of said at least one orthogonal pair of images, $(I_{h,x,z}, I_{h,y,z})$, the set, C_h, of possible sets of three-dimensional particle positions represented by x, y, and z image pixel values for each bead, b_h,

$C_h = (c_{h,1}, c_{h,2}, ...,c_{h,Eh})$, where $c_{h,e} = (x_{h,f,e}, y_{h,f,e}, z_{h,f,e})$,

4. where f = 1, 2, ..., F_h, and F_h being the number of particles of bead b_h, and e = 1, 2, ..., E_h, and E_h being the number of possible sets of three-dimensional particle positions for bead b_h,

5. deriving for each set of possible sets of three-dimensional particle positions one distance matrix

$$D_{h,e} = \begin{vmatrix} 0 & d_{h,e,1,2} & d_{h,e,1,3} & \dots & d_{h,e,1,Fh} \\ d_{h,e,2,1} & 0 & d_{h,e,2,3} & \dots & d_{h,e,2,Fh} \\ d_{h,e,3,1} & d_{h,e,3,2} & 0 & & \dots \\ \dots & \dots & & \dots & \\ d_{h,e,Fh,1} & d_{h,e,Fh,2} & \dots & & 0 \end{vmatrix}$$

where $d_{h,e,i,j} = integer([(x_{h,e,i}-x_{h,e,j})^2+(y_{h,e,i}-y_{h,e,j})^2+(z_{h,e,i}-z_{h,e,j})2]^{\frac{1}{2}})$, where $i = 1,2,...F_h$, and $j = 1,2,...,F_h$,

6. deriving for each distance matrix, $D_{h,e}$, the full set of derivable triangles, $T_{h,e} =$ ($t_{h,e,1}$, $t_{h,e,2}$, ..., $t_{h,e,Ghe}$), each triangle being represented by its three side length,

$T_{h,e} = [t_{h,e,1}, t_{h,e,2},..., t_{h,e,Ghe}] = [(d_{h,1,2}, d_{h,1,3}, d_{h,2,3}), (d_{h,1,2}, d_{h,1,4}, d_{h,2,4}),...,(d_{h,(Fh-2),(Fh-1)}, d_{h,(Fh-2),Fh}, d_{h,(Fh-1),Fh})]$,

$G_{h,e}$ being the total number of derivable triangles from distance matrix, $D_{h,e}$,

7. generating a subset, U, of all triangles, T, derived for the full set of beads, B, said subset of triangles comprising all different triangles derived for the full set of beads,

$U = (u_1, u_2, ..., u_W)$,

where $u_i \neq u_j$, for $i \neq j$, and $i = 1, 2, ..., W$, and $j = 1, 2, ..., W$, and W being the total number of different triangles derived for the full set of beads, B,

8. generating a look-up table, L, that for every triangle, u_r, where $r = 1, 2, ..., W$, gives the subset, A_r, of the full set of beads, B, for which subset of the full set of beads at least one of its derived sets of triangles comprises u_r,

$L = [(u_1, A_1), (u_2, A_2), ..., (u_W, A_W)]$,

9. obtaining at least one orthogonal pair of images ($I_{q,x,z}$, $I_{q,y,z}$) of the bead, b_q, to be identified,

10. deriving from said at least one orthogonal pair of images ($I_{q,x,z}$, $I_{q,y,z}$) the full set of possible sets of three-dimensional particle positions,

$$C_q = (c_{q,1}, c_{q,2}, \ldots c_{q,Eq}),$$

11. deriving for each of said sets of possible sets of three-dimensional particle positions one distance matrix

$$D_{q,e} = \begin{vmatrix} 0 & d_{q,e,1,2} & d_{q,e,1,3} & \ldots & d_{q,e,1,Fq} \\ d_{q,e,2,1} & 0 & d_{q,e,2,3} & \ldots & d_{q,e,2,Fq} \\ d_{q,e,3,1} & d_{q,e,3,2} & 0 & & \ldots \\ \ldots & \ldots & & \ldots & \\ d_{q,e,Fq,1} & d_{q,e,Fq,2} & \ldots & & 0 \end{vmatrix}$$

12. deriving for each distance matrix, $D_{q,e}$, the full set of derivable triangles, $T_q = (t_{q,e,1}, t_{q,e,2}, \ldots, t_{q,e,Gqe})$, each triangle being represented by its three side length,

$$T_q = [t_{q,e,1}, t_{q,e,2}, \ldots, t_{q,e,Gqe}] = [(d_{q,1,2}, d_{q,1,3}, d_{q,2,3}), (d_{q,1,2}, d_{q,1,4}, d_{q,2,4}), \ldots, (d_{q,(F-2),(F-1)}, d_{q,(F-2),F}, d_{q,(F-1),F})],$$

13. finding for each of said triangles of said set of triangles, T_q, derivable from bead b_q the corresponding set, B_q, of subsets of beads according to said look-up table, L, for which at least one of its derived sets of triangles comprises each of said triangles of said set of triangles, T_q, derivable from bead b_q,

14. registering for each of the beads of said subset of beads, B_q, the number of triangles contained in T_q,

15. identifying bead b_q as the bead of said subset of beads, B_q, that has the highest number of triangles contained in T_q.

When the phrase "all possible sets of particle positions" is being used herein above, it will be understood that based on at least one 2D projection, more than one 3D interpretation may exist due to correspondance problems as described elsewhere herein.

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Therefore, in one preferred embodiment of the present invention, "all possible sets of particle positions" shall mean "essentially all" possible set of particles positions, such as in more than about 90%, such as in more than 95% of all cases, "all possible sets of particle positions" shall mean "all sets of particle positions" for the bead in question.

The problem is that it may not always be possible to determine for each and every bead, the total number of geometrical figures for individual beads. As an example, when one particle is interfering with the imaging of another particle, e.g. by shielding another particle, it may not be possible to determine for such a shielded particle all coordinates x,y,z for a given microparticle.

Theoretical design criteria for identifying spatially encoded beads

In order to demonstrate the versatility of the above methods, theoretical design criteria for spatially encoded beads with optimal features for identification can be obtained by

1. Forming a virtual set of spatially encoded beads in a computer on the basis of a set of spatially encoded bead properties, e.g., spatially encoded bead size distribution, spatially immobilised particle size distribution, and number of spatially immobilised particles per macrobead. Also, optical parameters should be included in the analysis, especially the uncertainty involved in the determination of the spatially immobilised particle positions,
2. Simulating random rotation of all spatially encoded beads,
3. Computing one pair of orthogonal projections of each of the spatially immobilised particles of each spatially encoded bead,
4. Combining the two orthogonal sets of 2D-positions whereby the set of possible 3D-positions is obtained for each spatially encoded bead,
5. Computing the set of distance matrices corresponding to the set of 3D-positions thus determined,
6. Identifying single spatially encoded beads by comparing the full set of distance matrices of single spatially encoded beads against the full set of dis-

tance matrices of all spatially encoded beads. The best fit of single distance matrices hereby obtained identifies single spatially encoded beads.

7. Registering the number of not-identified spatially encoded beads,

8. Varying one or more spatially encoded bead parameters and repeating the sequence 1 to 7 a one or more times.

Finding theoretical encoded beads (EB) design criteria

A virtual set of N = 5000 spatially encoded beads was formed in a computer with the use of a MatLab code. The following input parameters were used:

Input parameter	Symbol	Value	Unit
EB diameter	D	800	micrometers
Spatially immobilised particle diameter	d	10	micrometers
Number of spatially immobilised particles per EB	M	5	-
Standard deviation of the error of the spatially immobilised particle positions	δ	4	micrometers

This virtual set of spatially encoded beads was fed to a MatLab code for multiple distance matrix identification, which gave rise to the following output parameters:

Output parameter	Symbol	Value	Unit
Number of spatially encoded beads with correspondence problem	C	458	
Number of ill-identified spatially encoded beads	E	165	

The number of spatially immobilised particles per spatially encoded bead, M, and the standard deviation of the error of the spatially immobilised particle positions, δ, were varied stepwise and fed to the multiple distance matrix ID code.

5   The result in terms of the number of spatially encoded beads with correspondence problem, C, and the number of ill-identified spatially encoded beads, E, is given in figure 11 where C and E are plotted against M and δ.

It can be seen from the upper plot of Fig. 11 that the number of correspondence
10   problems increases with increasing number of spatially immobilised particles and with the error associated with the determination of the relative spatially immobilised particle positions as one would expect.

The lower plot in Fig. 11 shows that in order to minimize the number of ill-identified
15   spatially encoded bead, each spatially encoded bead should preferably comprise from 4 to 6 spatially immobilised particles. However, other numbers are also possible, such as from 3 to 8 spatially immobilised particles, for example 3 or 4 spatially immobilised particles, such as from 6 to 8 spatially immobilised particles for example 3, 4, 5, 6, 7, or 8 spatially immobilised particles.

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At numbers of spatially immobilised particles below 4, the number of ill-identified spatially encoded beads increases abruptly, and at numbers higher than 4 spatially immobilised particles, the number of ill-identified spatially encoded beads increases gradually. The plot further shows that the number of ill-identified spatially encoded
25   beads gradually increases with the positional error, and that the method breaks down when the positional error involved in the determination increases from 6 to 8 micrometers. These results can be used as design parameters for generating individually identifiable, spatially encoded beads.

30   For finding the 2D spatially immobilised particle-positions in images with the use of MatLab Imaging toolbox, it is possible to use e.g. a number of Gauss models, such as from 6 to 8 Gauss models, with same shape and varying size are applied to the image. For each Gauss model applied, one goodness-of-fit images are generated with the use of linear filtering. A new image is generated on the basis of the good-
35   ness-of-fit images as pixelwise maximum of the goodness-of-fit images. The 2D

spatially immobilised particle-positions can be found in this image as the positions of local maxima that have a goodness-of-fit value above a pre-set threshold value.

Encoded bead reader device

In preferred embodiments of the present invention it is desirable to read distance-encoded synthesis beads at a high rate, i.e. reading at least 5000 spatially encoded beads per hour. The reading must result in data from which the distance matrix of individual spatially encoded beads can be extracted.

For this embodiment the present invention provides an encoded bead reader device which is described in more detail herein below.

In one preferred embodiment there is provided a device for recording and storing at least one image of at least one spatially encoded bead comprising a plurality of par-ticles, said device comprising i) at least one source of illumination, preferably a con-tinuous wave laser, ii) a flow cell comprising an imaging section, iii) at least one pulse generator, iv) at least one image intensifier, and v) at least one CCD camera, such as two or more CCD cameras. The device can further comprising a photo-sensor.

The encoded bead reader device comprise or be linked to a computer running a program for calculation of distance matrices for individual spatially encoded beads.

The photo-sensor for detecting entry of an encoded bead into the imaging section of the flow cell preferably comprises an optical objective for focussing said imaging section of said flow cell onto the photo-sensitive area of said photo-sensor. The op-tical objective of said photo-sensor preferably comprises a fluorescence filter for blocking the light of said laser, and the fluorescence filter is capable of transmitting the fluorescence emission from an individual encoded bead.

The CCD-camera(s) for recording at least one fluorescence image of an individual encoded bead preferably comprises a gated image intensifier for amplifying the fluo-rescence emission from the encoded bead. Each of the gated image intensifiers preferably comprises an optical objective for focussing said imaging section of said

flow cell onto the photo-sensitive area of each image intensifier. Each optical objective preferably comprises a fluorescence filter for blocking the light of said laser, and the fluorescence filter is capable of transmitting the fluorescence emission from an individual encoded bead.

The pulse generator can be an electrical square wave pulse generator for triggering said two or more CCD-cameras and/or said two or more image intensifiers.

It is preferred that the device further comprises an image storage system comprising one or more of the following elements: A framegrabber for recording the images from said two or more CCD-cameras, an electronic memory-device for storing said images from said framegrabber, a program code for controlling said electronic memory-device, and a computer for integrating said framegrabber and said electronic memory device and for executing said program code.

The encoded bead reader device can be used in methods for recording and optionally also storing images of individual spatially encoded beads. This is achieved by performing e.g. a method comprising the steps of

1. Dispersing spatially encoded beads in a liquid,
2. Diverting the dispersion of spatially encoded beads through a transparent flow cell,
3. Optionally detecting the coming of each spatially encoded bead with a photo-sensor, and
4. Recording one pair of orthogonal fluorescence images of each spatially encoded bead, and
5. Optionally storing the images of each spatially encoded bead on a computer,

For the above purpose, the encoded bead reader device preferably comprises a flow system comprising a flow cell, an imaging system, and optionally also an image storage system;

wherein the flow system in detailed embodiments comprises

1. A flask for containing a set of EB in a liquid, said flask being equipped with a magnetic stirrer for dispersing the EB in said liquid

2. A syringe pump for pumping the EB, said syringe pump being equipped with magnetic stirrers for keeping the EB dispersed inside the syringes of said syringe pump, and said syringe pump being equipped with an automatic four-way valve for ensuring one-way flow.

3. A tube connecting said flask with said syringe pump

4. A transparent flow cell with a rectangular cross section, said flow cell having an imaging section for imaging of EB

5. A tube connecting said syringe pump with said flow cell

6. Two or more reservoirs

7. An exit tube connecting said flow cell to one of said two or more reservoirs, said exit tube having an exit tube outlet

8. A switch for controlling which one of said two ore more reservoirs is connected to said exit tube outlet;

wherein the imaging system comprises

1. A continuous wave laser for illuminating said imaging section of said flow cell

2. A photo-sensor, such as a photo-multiplier, for detecting when an EB enters said imaging section of said flow cell, said photo-sensor being equipped with an optical objective for focussing said imaging section of said flow cell onto the photo-sensitive area of said photo-sensor, and said optical objective of said photo-sensor being equipped with a fluorescence filter for blocking the light of said laser, and said fluorescence filter transmitting the fluorescence emission from the EB

3. Two or more video-cameras for obtaining fluorescence images of the EB, each one of said two or more video-cameras being equipped with one gated image intensifier for amplifying the fluorescence emission from the EB, and each one of said image intensifiers being equipped with one optical objective for focussing said imaging section of said flow cell onto the photo-sensitive area of each image intensifier, and each optical objective being equipped with one fluorescence filter for blocking the light of said laser, and said fluorescence filter transmitting the fluorescence emission from the EB

4. An electrical square wave pulse generator for triggering said two or more cameras and said two or more image intensifiers

5. An electrical cable connecting the output terminal of said photo-sensor to the trigger input of said pulse generator whereby it is obtained that a square wave pulse is generated when the output voltage of said photo-sensor is above the trigger-voltage of said pulse generator

6. Electrical cables for connecting the output terminal of said pulse generator to the input terminals of said two or more video-cameras and said two or more image intensifiers, whereby it is obtained that two simultaneous images are recorded with said two cameras; and

wherein the optional image storage system comprises

1. A framegrabber for recording the images from said two or more cameras

2. An electronic memory-device for storing said images from said framegrabber

3. A program code for controlling said electronic memory-device

4. A computer for integrating said framegrabber and said electronic memory device and for executing said program code

In one preferred embodiment, the imaging system of the encoded bead reader device comprises:

a) a quartz flow cell comprising cylindrical entrance and exit sections of length 25mm, inner diameter 1mm, and outer diameter 3mm, a central section between said entrance and exit sections of length 10mm, inner rectangular cross section 1mm x 1mm and outer rectangular cross section 3mm x 3mm, and an approximately cubic imaging section of said central section of inner dimensions ca. 1mm x 1mm x 1mm, the exact position and geometry of said imaging section being determined by the source of illumination,

b) a silicone rubber tube connected to said entrance section of said flow cell for feeding an encoded beads suspension to said flow cell,

c) a silicone rubber tube connected to said exit section of said flow cell for removing an encoded beads suspension from said flow cell,

d) a continuous wave laser (e.g. the BluePoint series supplied by Rainbow Photonics, the Cobolt Blue series supplied by Cobolt AB, or the Blue CrystaLaser series supplied by Crysta Laser) of wavelength 473 nm for illuminating the imaging

section of said central section of said flow cell and for controlling the position and geometry of said imaging section of said central section of said flow cell,

e)   two CCD cameras (e.g. the CPL high speed series supplied by Canadian Photonics Labs Inc., the SR-CMOS series supplied by Vision Research, or the SVS series supplied by SVS-Vistek GmbH), a first and a second CCD camera, being positioned perpendicular to each other and aligned relative to said imaging section of said central section of said flow cell in such a way that the CCD chips of said CCD cameras run parallel to the flat surfaces of said central section of said flow cell and so that said imaging section of said central section of said flow cell can be projected onto said two CCD chips of said two CCD cameras by optical means described below,

f)   two image intensifiers (such as supplied by Hamamatsu or the Proxifier series supplied by Proxitronic, or the GPM series supplied by Photonicstech) connected to said two CCD cameras for amplifying the optical signal emitted from the illuminated section of said imaging section of said flow cell,

g)   a photo-multiplier for detecting the coming of an encoded bead, said photo-multiplier being positioned opposite to first of said two CCD cameras and perpendicular to second of said two CCD cameras and aligned relative to said imaging section of said central section of said flow cell in such a way that the photosensitive area of said photo-multiplier runs parallel to the CCD chip of said first of said two CCD cameras and perpendicular to the CCD chip of said second of said two CCD cameras,

h)   three objectives (such as the MS-50 supplied by MEIJI TECHNO or the QM-100 supplied by Questar), a first, a second, and a third objective, said first and second objectives of said three objectives being connected to said first and second CCD cameras, whereby the optical signal from said imaging section of said central section of said flow cell is focussed onto said two CCD chips of said two CCD cameras, and said third objective of said three objectives being connected to said photo-multiplier, whereby it is obtained that the optical signal from said imaging section of said central section of said flow cell is focussed onto said photosensitive area of said photo-multiplier,

i)   three optical filters (emmision band pass filters, e.g. type 528-50 supplied by Ferroperm or Chroma) connected to said three objectives for blocking the laser light and transferring the fluorescence emmission from the fluorescent spatially immobilised particles of the encoded beads described elsewhere,

j)   an electronic amplifier connected to the electric output terminals of said photo-
multiplier for amplifying the electrical output from said photo-multiplier,

k)   an electronic pulse generator (type TGP110 supplied by Thurlby Thanders in-
struments, TT*i*) for generating a pulse for simultaneous triggering of said two

5   cameras and said two image intensifiers, whereby it is obtained that simultane-
ous pairs of images can be recorded with said two CCD cameras, the input ter-
minals of said pulse generator being connected to the output terminals of said
electronic amplifier, whereby it is obtained that images are recorded only when
one or more spatially immobilised particles of an encoded bead passes through

10   said imaging section of said central section of said flow cell,

l)   a framegrabber (type GrabLink Expert supplied by Eurecard) connected to the
output terminals of said two CCD cameras for transferring the electronic signals
from said two CCD cameras to a computer, and

m) a personal computer (PC type Pentium 4 supplied by Unit-One electronics) con-
15   nected to the output terminals of said framegrabber for electronically storing the
images from said two CCD cameras.

As an alternative to the use of CCD cameras any suitable digital camera can be
used, e.g., C-MOS cameras.

20

As an alternative to the use of image intensifiers connected to CCD cameras, on-
chip multiplication gain cameras can be used.

In a further embodiment of the encoded bead reader device the image processing of
25   each pair of orthogonal images, the calculation of the set of possible 3D particle
positions, and the corresponding set of possible distance matrices is preferably car-
ried out on-line by a fast computer, i.e. a computer capable of performing the above
operations in less than 0.5 sec.

30   In yet another embodiment of the encoded bead reader device the image process-
ing of each pair of orthogonal images is carried out on-line by a fast computer and
the number of particles of each encoded bead is determined. Furthermore, the en-
coded bead reader device can comprise a fast switching valve positioned down-
stream from the flow cell and means for controlling said valve on the basis of the

35   number of particles per encoded bead, whereby encoded bead sorting according to

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number of particles per encoded bead is enabled. Preferably encoded beads with 4 – 8 particles are separated. Hereby a set of encoded beads with 4 – 8 particles per encoded bead is obtained.

5   One embodiment of the encoded bead reader includes an illumination flow cell positioned up-stream from the imaging flow cell. When phosphorescent particles leave the illumination flow cell they emit light and can be imaged in the imaging flow cell. In this embodiment emission filters are not required.

10   Beaded polymer matrix

It is one object of the present invention to provide an encoded, beaded or granulated polymer matrix for solid phase synthesis in the form of a bead or a granule comprising a plurality of spatially immobilised particles or vacuoles, wherein each particle or
15   vacuole is individually detectable. The beaded matrix has different optical or spectroscopic properties from those of the immobilised particles or vacuoles. The immobilised particles or vacuoles can be monodisperse or heterodisperse, and the immobilised particles can be regular spherical beads or vacuoles, or they can be irregular particles. The beaded polymer matrix can be spherical, i.e. having a regular,
20   rounded shape, or it can have an irregular shape in the form of a granule.

Each beaded or granulated polymer matrix preferably comprises at least 2 particles, such as at least 3 particles, for example at least 4 particles. The particles can be essentially spherical, and preferably at least 2 such as 3, for example 5 of said parti-
25   cles have essentially the same diameter. The particles are preferably essentially monodisperse and/or less than 10 micrometer in diameter, such as less than 5 micrometer in diameter, for example less than 1 micrometer in diameter, such as less than 0.1 micrometer in diameter.

30   The present invention resides in one embodiment in a bead on which a compound can be synthesised, wherein the bead has at least two markers integrally associated therewith, which markers are detectable and/or quantifiable during synthesis of the compound. The markers define a code identifying the bead before, during and after synthesis of a compound. Through the use of its plurality of detectable and/or quan-
35   tifiable markers, preferably optically detectable and/or quantifiable markers, the

bead of the present invention provides more "pre-encoded" information compared to other beads of the prior art and thus provides larger combinational library sizes that can be encoded.

This "pre-encoded" information may be read by specialized apparatus such as e.g. flow cytometers and the information can be used to track the synthetic history of an individual bead in a combinatorial process as described hereinafter. An example of a specialised apparatus for recording the "pre-encoded" information contained in an encoded bead is the specialised encoded bead reader apparatus disclosed herein below.

The larger the diversity of detectable and/or quantifiable markers of a bead, the greater the degree of decipherability or resolution of the bead in a large population of beads. In this regard, each detectable and/or quantifiable marker of a bead provides at least a part of the information required to distinctively identify the bead. The larger the number of such markers, the more detailed the identifying information that is compilable for a given bead, which may be used to distinguish that bead from other beads. On the other hand the complication of detection is increased markedly with the number of markers.

Markers

The particles can comprise a marker, which is detectable by any form of electromagnetic radiation including fluorescent emmision. However, the marker can also be detected by fast spectroscopic techniques other than fluorescence spectroscopy. The particles of said beaded or granulated polymer matrix in one embodiment comprise a spectroscopically detectable marker and/or a fluorescently detectable marker.

Fluorescently detectable markers are preferably selected from the fluorescent group of compounds and materials consisting of fluorencent organic polycyclic compounds, conjugated vinylic compounds, heterocyclic transition metal complexes, rare earth metal compounds, inorganic oxides and glasses.

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Fluorescently detectable markers can be detected by two photon fluorescence spectroscopy and/or by one photon fluorescence spectroscopy. Fluorescently detectable markers can additionally be detected by time-correlated photon fluorescence spectroscopy.

Examples of detection by fast spectroscopic techniques other than fluorescence spectroscopy include, but is not limited to fast spectroscopic techniques such as infrared spectroscopy, raman spectroscopy, visble light spectroscopy, UV spectroscopy, electron spin resonance, and nuclear magnetic resonance.

The fluorescently detectable marker is preferably selected from the group consisting of dyes based on the structure of fluorescein, oregon green, rhodamine, aminobenzoic acid, Alexa™ probes, BODIPY-dyes, cascade blue dye, coumarine, naphthalenes, dansyl, indoles, pyrenes pyridyloxazole, cascade yellow dye, Dapoxyl Dye, Fluorescamine, aromatic ortho dialdehydes, OPA and NDA, ATTO-Tag's, 7-Nitrobenz-2-Oxa-1,3-Diazole or derivatives thereof. The fluorescently detectable marker in one embodiment is preferably a UV or visible light–excitable microsphere.

Fluorescent dyes may be incorporated into beads by any suitable method known in the art, such as copolymerisation of a polymerisable monomer and a dyecontaining co-monomer or addition of a suitable dye derivative in a suitable organic solvent to an aqueous suspension as for example disclosed in Singer et al., (supra including references cited therein), Campian et al. (1994, In"Innovation and Perspectives on Solid Phase Synthesis"Epton, R., Birmingham: Mayflower, 469-472, incorporated herein by reference) and Egner et al. (1997, Chem. Commun. 73 5-73 6, incorporated herein by reference). Alternatively, fluorescent beads may be produced having at least one fluorescent spherical zone. Such particles may be prepared as for example described in U. S. Patent No. 5,786,219 (Zhang et al.), which is incorporated herein by reference. In a preferred embodiment, one or more fluorescent dyes are incorporated within a microparticle. Compared to surface attachment of fluorescent dyes, incorporation of dyes within beads reduces the physical exposure of the fluorescent dye (s) to various solvents used in combinatorial synthesis and thus increases the stability of the beadfluorescent dye complexes.

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Also included in the present invention are markers which are detectable by fast detection techniques other than spectroscopy, such as light scattering, reflection, diffraction or light rotation.

5   Electromagnetic radiation-related markers are preferably selected from the group consisting of fluorescence emission, luminescence, phosphorescence, infrared radiation, electromagnetic scattering including light and X-ray scattering, light transmittance, light absorbance and electrical impedance.

10   Preferably, the electromagnetic radiation-related marker is a light emitting, light transmitting or light absorbing marker detectable by illuminating the particle with incident light of one or more selected wavelengths or of one or more selected vectors.

15   It is preferred that at least one of the markers of a bead is an electromagnetic radiation-related marker suitably selected from the group consisting of atomic or molecular fluorescence emission, luminescence, phosphorescence, infrared radiation, electromagnetic scattering including light and X-ray scattering, light transmittance, light absorbance and electrical impedance.

20

The fluorescence emission can result from e.g. excitation of one or more fluorescent markers attached to, or contained within, the bead. In the case of two or more fluorescent markers being utilised, the markers can be the same and the markers can comprise the same or varying amounts of a fluorophore. In the latter case the mark-

25   ers are intensity-differentiated.

Alternatively, the markers may be different wherein they are present in a ratio of 1: 1 or varying ratios. Reference may be made in this regard to WO 95/32425 which is incorporated herein by reference.

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Exemplary fluorophores which may be used in accordance with the present invention include those listed in WO 93/06121, which is incorporated by reference herein.

Any suitable fluorescent dye may be used for incorporation into the bead of the in-

35   vention. For example, reference may be made to U. S. Patents 5,573,909 (Singer et

al., which is incorporated herein by reference) and 5,326,692 (Brinkley et al., which is incorporated herein by reference) which describe a plethora of fluorescent dyes. Reference may also be made to fluorescent dyes described in U. S. Patent Nos. 5,227,487, 5,274,113, 5,405,975, 5,433,896, 5,442,045, 5,451,663, 5,453,517,

5   5,459,276, 5,516,864, 5,648,270 and 5,723,218, which are all incorporated herein by reference.

In one embodiment, one or more of the fluorescent markers can preferably be incorporated into a microparticle, such as a polymeric microparticle, or a ceramic mi-

10   croparticle. Such microparticles can preferably be attached to a bead by use of e.g. colloidal interactions as for example disclosed by Trau and Bryant in PCT/AU98/00944, incorporated herein by reference.

When the marker is spectroscopically detectable, there is in one embodiment pro-

15   vided a marker capable of being probed by a range of frequencies differing by less than about 20%, such as less than about 10%, based on the numerical highest frequency value. The marker can also be probed by one or more predetermined frequencies.

20   Any suitable method of analysing fluorescence emission is encompassed by the present invention. In this regard, the invention contemplates techniques including, but not restricted to, 2-photon and 3-photon time resolved fluorescence spectroscopy as for example disclosed by Lakowicz et al. (1997, Biophys. J., 72: 567, incorporated herein by reference), fluorescence lifetime imaging as for example disclosed

25   by Eriksson et al. (1993, Biophys. J., 2: 64, incorporated herein by reference), and fluorescence resonance energy transfer as for example disclosed by Youvan et al. (1997, Biotechnology et alia 3: 1-18).

Luminescence and phosphorescence may result respectively from a suitable lumi-

30   nescent or phosphorescent label as is known in the art. Any optical means of identifying such label may be used in this regard.

Infrared radiation may result from a suitable infrared dye. Exemplary infrared dyes that may be employed in the invention include, but are not restricted to, those dis-

35   closed in Lewis et al. (1999, Dyes Pigm. 42 (2): 197), Tawa et al. (1998, Mater. Res.

Soc. Symp. Proc. 488 (Electrical, Optical, and Magnetic Properties of Organic Solid-State Materials IV), 885-890), Daneshvar, et al. (1999, J. Immunol. Methods 226 (1-2): 119-128), Rapaport et al. (1999, Appl. Phys. Lett. 74 (3): 329-331) and Durig et al. (1993, J. Raman Spectrosc. 24 (5): 281-5), which are incorporated herein by ref-

5   erence. Any suitable infrared spectroscopic method may be employed to interrogate the infrared dye. For instance, fourier transform infrared spectroscopy as for example described by Rahman et al. (1998, J. Org. Chem., 63: 6196, incorporated herein by reference) may be used in this regard.

10   Suitably, electromagnetic scattering may result from diffraction, reflection, polarisation or refraction of the incident electromagnetic radiation including light and Xrays. In this regard, the beads may be formed of different materials to provide a set of beads with varying scattering properties such as different refractive indexes as for example described supra. Any suitable art recognised method of detecting and/or

15   quantifying electromagnetic scatter may be employed. In this regard, the invention also contemplates methods employing contrast variation in light scattering as, for example, described in van Helden and Vrij (1980, Journal of Colloidal and Interface Science 76: 419-433), which is incorporated herein by reference.

20   Markers other than electromagnetic radiation-related markers can be utilised, optionally in combination with electromagnetic radiation-related markers. Such markers include e.g. size and/or shape of the bead. For example, beads may be shaped in the form of spheres, cubes, rectangular prisms, pyramids, cones, ovoids, sheets or cylinders, including intermediate forms as well as irregular shapes. Electrical imped-

25   ance across a bead may be measured to provide an estimate of the bead volume (Coulter).

The marker in one embodiment comprises a chromophoric label. Suitable beads comprising such chromophores are described e.g. by Tentorio et al. (1980, Journal

30   of Colloidal and Interface Science 77: 419-426), which is incorporated herein by reference.

A suitable method for non-destructive analysis of organic pigments and dyes, using a Raman microprobe, microfluorometer or absorption microspectrophotometer, is

35   decribed for example in Guineau, B. (1989, Cent. Rech. Conserv. Documents

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Graph., CNRS, Paris, Fr. Stud. Conserv 34 (1): 38-44), which is incorporated herein by reference.

Alternatively, the marker may comprise a magnetic material inclusive of iron and magnetite, or an marker that is detectable by acoustic backscatter as is known in the art.

It will be understood from the foregoing that the number of beads having different detectable codes will be dependent on the number of different detectable and/or quantifiable markers integrally associated with the beads.

Beads, such as polymer beads and other types of beads

Polymers according to the present invention are preferably optically transparent in the optical exitation range of the fluorescent marker and/or the emission wavelength range of the fluorescent marker comprised by the particles and/or vacuoles of the polymer matrix.

Polymer beads according to the invention can be prepared from a variety of poly-merisable monomers, including styrenes, acrylates and unsaturated chlorides, es-ters, acetates, amides and alcohols, including, but not limited to, polystyrene (includ-ing high density polystyrene latexes such as brominated polystyrene), polymethyl-methacrylate and other polyacrylic acids, polyacrylonitrile, polyacrylamide, polyac-rolein, polydimethylsiloxane, polybutadiene, polyisoprene, polyurethane, polyvi-nylacetate, polyvinylchloride, polyvinylpyridine, polyvinylbenzylchloride, polyvinyl-toluene, polyvinylidenechloride and polydivinylbenzene, as well as PEGA, SPOCC and POEPOP. The beads may be prepared from styrene monomers or PEG based marcromonomers.

The polymer is in preferred embodiments selected from the group consisting of polyethers, polyvinyls, polyacrylates, polymethacrylates, polyacylamides, polyure-thanes, polyacrylamides, polystyrenes, polycarbonates, polyesters, polyamides, and combinations threreof.

In more preferred embodiments, the polymer is selected from the group consisting of SPOCC, PEGA, HYDRA, POEPOP, PEG-polyacrylate copolymers, polyether-polyamine copolymers, crosslinked polyethylene diamines, and combinations thereof.

However, the invention is not limited to the above polymers as the beads can in principle comprise any solid, at least partly transparent material capable of providing a base for combinatorial synthesis. As illustrative examples, the beads can be polymeric supports such as polymeric beads, which are preferably formed from polystyrene cross-linked with 1-5% divinylbenzene. Polymeric beads can also be formed from hexamethylenediaminepolyacryl resins and related polymers, poly N- {2- (4-hydroxylphenyl) ethyl} acrylamide (i. e., (one Q)), silica, cellulose beads, polystyrene beads poly (halomethylstyrene) beads, poly (halostyrene) beads, poly (acetoxystyrene) beads, latex beads, grafted copolymer beads such as polyethylene glycol/polystyrene, porous silicates for example controlled pore-glass beads, polyacrylamide beads for example poly (acryloylsarcosine methyl ester) beads, dimethylacrylamide beads optionally cross-linked with N, N'-bis-acrylolyl ethylene diamine, glass particles coated with a hydrophobic polymer inclusive of cross-linked polystyrene or a fluorinated ethylene polymer which provides a material having a rigid or semi-rigid surface, poly (N-acryloylpyrrolidine) resins, Wang resins, Pam resins, Merrifield resins, PAP and SPARE polyamide resins, polyethylene functionalised with acrylic acid, kieselguhr/polyamide (Pepsyn K), polyHipeTM, PS/polydimethylacrylamide copolymers, CPG, PS macrobeads and TentagelTM, PEG-PS/DVB copolymers.

Ceramic beads may be comprised of silica, alumina, titania or any other suitable transparent material. A suitable method of making silica beads is described, for example in"The Colloid Chemistry of Silica and Silicates" (Cornell University Press) by Ralph K Iler 1955 and U. S. Patent No 5,439,624, the disclosures of which are incorporated herein by reference. Reference may also be made to WO95/25737 and WO97/15390, incorporated herein by reference, which disclose examples of such beads.

The beaded polymer matrix according to the invention preferably has a ratio R = a/b between a) the volume of the beaded or granulated polymer matrix and b) the aver-

age volume of the particles which is in the range of from 10000000:1 to 10:1, such as in the range of from 1000000:1 to 30:1, for example in the range of from 1000000:1 to 100:1, for example in the range of from 1000000:1 to 200:1, such as in the range of from 1000000:1 to 1000:1, for example in the range of from 100000:1 to
5   1000:1, such as in the range of from 100000:1 to 2000:1.

Independently of the above ratios, the beaded or granulated polymer matrix according to the invention preferably comprises an average volume of the swelled bead or granule of from 0.000001 µL – 50 µL, such as an average volume of the swelled
10   bead or granule of from 0.00001 µL – 5 µL, for example an average volume of the swelled bead or granule of from 0.001 µL – 1 µL, such as an average volume of the swelled bead or granule of from 0.01 µL – 0.1 µL.

Any combination of the above falls within the invention and accordingly, for a ratio R
15   = a/b between a) the volume of the beaded or granulated polymer matrix and b) the average volume of the particles which is in the range of from 10000000:1 to 10:1, such as in the range of from 1000000:1 to 30:1, for example in the range of from 1000000:1 to 100:1, for example in the range of from 1000000:1 to 200:1, such as in the range of from 1000000:1 to 1000:1, for example in the range of from 100000:1 to
20   1000:1, such as in the range of from 100000:1 to 2000:1, the average volume of the swelled bead or granule can be from 0.000001 µL – 50 µL, such as an average volume of the swelled bead or granule of from 0.00001 µL – 5 µL, for example an average volume of the swelled bead or granule of from 0.001 µL – 1 µL, such as an average volume of the swelled bead or granule of from 0.01 µL – 0.1 µL.
25

Composition comprising a plurality of encoded, beaded polymer matrices

The invention is in one embodiment directed to a plurality of beads comprising a population that is pre-encoded. Accordingly, each bead of that population has a
30   code, which distinctively identifies a respective bead before, during and after said synthesis from other beads. The diversity of the said population of beads, therefore, resides in beads of said population having relative to each other different spatial locations of detectable markers, which are used to provide distinctive codes for detection of each of those beads.
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The composition of beads of the invention may be used in many applications, such as affinity chromatography for purification and/or isolation of desirable target compounds, and combinatorial chemistry procedures that do or do not involve a split-and-combine procedure. Preferably, however, such assemblies are used in combinatorial chemistries, which involve a split-process-recombine procedure.

A plurality of beads according to the invention may be prepared by any suitable method. Preferably, when colloidal particles including polymeric and ceramic particles are used as beads, the colloid dispersion of such beads is stabilised. Exemplary methods imparting colloidal stabilisation are described for example in Hunter, R. J. (1986, "Foundation of Colloid Science", Oxford University Press, Melbourne) and Napper, D. H. (1983,"Polymeric stabilisation of Colloidal Dispersions"Academic Press, London), the disclosures of which are incorporated herein by reference. In this regard, the most widely exploited effect of nonionic polymers on colloid stability is steric stabilisation, in which stability is imparted by polymer molecules that are absorbed onto, or attached to, the surface of the colloid particles. Persons of skill in the art will recognise that it is possible to impart stability by combinations of different stabilisation mechanisms: e. g., surface charge on the particles can impact colloidal stability via electrostatic stabilisation, and an attached polyelectrolyte can impart stability by a combination of electrostatic and steric mechanisms (electrosteric stabilisation). Polymer that is in free solution can also influence colloid stability. Stabilisation by free polymer is well-documented (Napper 1983, supra) and is called depletion stabilisation.

Preferably, steric stabilisation of colloid dispersions is employed. In this regard, steric stabilisation is widely exploited because it offers several distinct advantages over electrostatic stabilisation. For example, one advantage is that aqueous sterically stabilised dispersions are comparatively insensitive to the presence of electrolytes because the dimensions of non-ionic chains vary relatively little with the electrolyte concentration.

Any suitable stabilising moiety may be used for stabilising colloidal dispersions. Exemplary stabilising moieties that impact on colloidal stability are given herein below: Poly (oxyethylene), Poly (vinyl alcohol), Poly (acrylic acid), Poly (acrylamide), and sorbitol monolaurate as well as commonly used emulsion stabilizers.

The composition of encoded, beads preferably comprises at least 10^2 individually identifiable beads, such as at least 10^3 individually identifiable beads, for example at least 10^5 individually identifiable beads, such as at least 10^7 individually identifiable beads, for example at least 10^9 individually identifiable beads, such as at least 10^{11} individually identifiable beads, for example at least 10^{13} individually identifiable beads, such as at least 10^{15} individually identifiable beads, for example at least 10^{17} individually identifiable beads, such as at least 10^{19} individually identifiable beads, for example at least 10^{21} individually identifiable beads, such as at least 10^{23} individually identifiable beads.

Methods for generating a composition comprising encoded beads

It is a further object of the invention to provide a method for generating a composition comprising a plurality of encoded, beaded polymer matrices, said method comprising the steps of

i)   synthesizing a monomer and/or macromonomer and a crosslinker for polymerization, and,

ii)   mixing the monomer and/or macromonomer with particles to give an even dispersion of particles in the mixture, and

iii)   polymerizing the monomer and/or macromonomer by either i) suspension polymerisation and/or; ii) inverse suspension polymerisation and/or iii) bulk polymerisation followed by granulation and/or iv) droplet polymerisation.

In a further aspect there is provided a method for generating a composition comprising a plurality of encoded, beaded polymer matrices, and detecting and/or identifying individually identifiable beads, said method comprising the steps of:

(a) preparing a plurality of beads comprising spatially immobilised particles comprising at least one marker;

(b) detecting and/or quantifying the said markers of each bead and assigning a code, such as the result of a determination of the location of spatially encoded parti-

cles or vacuoles, for each bead;

(c) identifying beads having distinctive codes; and optionally

(d) identifying beads having similar codes; and further optionally

(e) sorting the beads having distinctive codes from the beads having non
5 distinctive codes to thereby provide an encoded, beaded polymer matrix.

There is also provided the use of such a composition comprising a plurality of en-
coded, beaded polymer matrices linked to a bioactive compound for identifying bio-
active compound binding partners, and a use of the composition of beads linked to
10 different bioactive compounds for diagnostic purposes, wherein the binding and de-
termination of a predetermined binding partner to a substrate or bioactive compound
on the carrier is at least indicative of a positive diagnosis.

Bioactive compounds of particular interest are e.g. those which may be so screened
15 include agonists and antagonists for cell membrane receptors, toxins, venoms, viral
epitopes, hormones, sugars, co-factors, peptides, enzyme substrates, drugs inclu-
sive of opiates and steroids, proteins including antibodies, monoclonal antibodies,
antisera reactive with specific antigenic determinants, nucleic acids, lectins, poly-
saccharides, cellular membranes and organelles.
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The present invention also encompasses as bioactive compounds a plurality of
unique polynucleotide or oligonucleotide sequences for sequence by hybridisation
(SBH) or gene expression analyses. Persons of skill in the art will recognise that
SBH uses a set of short oligonucleotide probes of defined sequence to search for
25 complementary sequences on a longer target strand of DNA. The hybridisation pat-
tern is used to reconstruct the target DNA sequence. Accordingly, in the context of
the present invention, an aqueous solution of fluorescently labelled single stranded
DNA (ssDNA) of unknown sequence may be passed over the library of polynucleo-
tide or oligonucleotide compounds and adsorption (hybridisation) of the ssDNA will
30 occur only on beads which contain polynucleotide or oligonucleotide sequences
complementary to those on the ssDNA. These beads may be identified, for example,
by flow cytometry, fluorescence optical microscopy or any other suitable technique.

Once a compound having the desired activity is obtained, the sequence of reaction
35 steps experienced by the bead on which the compound was synthesised may be

deconvoluted simply by analysing the tracking data for that bead as described, for example, hereinafter. The sequence of building blocks defining the compound of interest may thus be ascertained and a molecule comprising this sequence can by synthesised by conventional means (e. g., amino acid synthesis or oligonucleotide synthesis) as is known in the art.

Encoded beads can be sorted according to at least one optical parameter, i.e., a physical property that influences the optical signal arising from the encoded bead, such as size and shape and number of particles per encoded bead, whereby it is obtained that the resulting encoded beads are individually identifiable by optical means. Preferred means for sorting encoded beads include sedimentation, centrifugation, sieving, cyclone separation, total fluorescence separation, and separation according to number of particles per encoded bead. Total fluorescence separation can be carried out on a so-called bead sorter, such as the COPAS™ system supplied by Union Biometrica. Separation according to number of particles per encoded bead can be carried out with an encoded bead reader, such as disclosed above, equipped with a fast image processing system for counting the number of particles per encoded bead on the basis of the images and further equipped with a switchable valve positioned downstream from the flow cell for sorting the encoded beads.

It is essential that that the optical properties of said particles differ from the optical properties of said beaded polymer matrix, whereby it is obtained that the relative positions of said particles of said encoded beaded polymer matrix can be determined.

The polymerisation reaction can preferably be a radical initiated chain polymerisation reaction, or an anion initiated ring opening polymerisation reaction, or a cation initiated ring opening polymerisation reaction.

Functional groups on the beads can subsequently be reacted with different bioactive compound building blocks as described herein elsewhere. Each reaction step can be monitored as essentially each bead of the encoded, beaded polymer matrix is individually detectable. The below methods describe in more detail the identification of spatially immobilised particles or beads in the beads or granules.

51

Sorting spatially immobilised particles according to size and controlling the size distribution of spatially immobilised particles.

Particles may be sorted according to at least one optical parameter, i.e., a physical property that influences the optical signal arising from the particle, such as size, shape, colour, or fluorescence, whereby it is obtained that the relative positions of said particles can be determined by optical means. Preferred means for sorting particles include sedimentation, centrifugation, sieving, and cyclone separation.

Using spatially immobilised particles as particles in encoded beads places some limitations on the size of the spatially immobilised particles. Too large spatially immobilised particles tens to shadow each other and too small spatially immobilised particles may pass through the optical set-up unnoticed. Generally the size distribution of spatially immobilised particles synthesized by suspension polymerisation or emulsion polymerization is very broad. Hence, a method for obtaining a fraction of spatially immobilised particles with controlled size distribution is required.

It has been found that the micro beads in di-methylformamide (DMF) solution after centrifugation at 250rpm for 22min are considerably smaller than the micro beads in the sediment. This indicates that it is possible to remove small beads by repeated centrifugation at 250rpm and removing the liquid phase after each run, i.e. the concentration of small beads in the sediment should decrease after each run. Figure 13 shows micrographs of suspension polymerised micro beads before centrifugation and after 5 times centrifugation at 250rpm for 22min. A change in the size distribution towards a more narrow distribution and a higher average micro bead diameter appears from the micrographs. The size distribution was measured within a rectangular section of each image with the use of imaging software. Figure 14 shows the measured size distributions. It is clear from the figure that a more narrow size distribution and a larger average diameter are obtained by the method.

Method for identifying individual spatially encoded, beads in a composition comprising such spatially encoded beads

In yet another embodiment, the present invention provides a method for identifying at least one individually identifiable, spatially encoded beaded polymer matrix, said method comprising the steps of

5   i)   determining the unique, spatial immobilisation of a plurality of particles in the at least one bead to be identified, and

     ii)   identifying said at least one individually identifiable, spatially encoded beaded polymer matrix based on said unique determination of said spatially

10   immobilised plurality of particles.

Post identification of spatially encoded beads

The sequence comprising 1) determination of the spatially immobilised particle positions in the images, 2) calculation of the corresponding set of possible 3D-positions of the spatially immobilised particles and the corresponding distance matrices, and 3) the distance matrix based identification, may be too time consuming to allow for on-line identification of encoded beads. Instead post identification of "hits", i.e., spatially encoded beads carrying compounds which are of interest in a given assay, as illustrated in Fig. 15.

As the hits are not identified until after the full combinatorial chemistry synthesis, the hit ID will have to be carried out after the combinatorial synthesis process has been finished. Following a procedure comprising the following steps can do this:

1. A plurality of spatially encoded polymer beads is synthesized

2. Images or laser scans of each encoded bead is recorded and stored as the beads are being split into a number of jars, J_1, J_2... J_i in which jars one combinatorial synthesis step is carried out.

3. All beads are pooled

4. The sequence comprising steps 2 and 3 is repeated a number of times.

5. All spatially encoded beads are screened in a given assay and the *hits* are separated

6. The jar sequence of each *hit* is determined on the basis of the recordings obtained under step 2 and the use of an ID method.

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Method for generating an encoded, beaded polymer matrix comprising different bio-active compounds

5   It is a yet further object of the invention to provide a method for generating an en-
coded, beaded polymer matrix comprising a bioactive compound, wherein essen-
tially each bead of the polymer matrix is individually identifiable, said method com-
prising the steps of

10   spatially immobilizing particles in polymer beads or granulates, and

isolating encoded beads or granules by automated sorting, and

recording and storing the distance matrix for essentially each bead or granule, and

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performing a stepwise synthesis of bioactive compounds by reacting functional
groups of the encoded beads or granules with at least one building block, and

recording the identity of each bead or granule that enter each reaction step iv), and

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isolating beads or granules of interest, preferably by performing an assay or a diag-
nostic screen, and

identifying the bioactive compound attached to at least one individual bead by re-
25   cording the identity of at least one isolated bead or granule, and optionally compar-
ing said recording with the recording, preferably a distance matrix, recorded for at
least a plurality of the remaining beads or granules.

A binding assay for characterising or isolating bioactive compounds bound to the
30   beads or granules can be performed by measuring e.g. the binding of a protein to a
ligand bound to the polymer matrix. Also, an assay can be performed by measuring
e.g. an enzyme activity on a substrate bound to the polymer matrix. It is also possi-
ble to perform an assay by measuring e.g. enzyme inhibition of a molecule bound to
the polymer matrix, or to perform an assay by measuring e.g. receptor interaction
35   with a bioactive compound bound to the polymer matrix.

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For the above methods, the plurality of particles preferably comprise a fluorescently detectable marker, such as a fluorescently detectable marker detectable by two photon fluorescence microscopy, or a fluorescently detectable marker detectable by one photon fluorescence microscopy.

Method for deconvoluting a conventional library

In a further aspect, the invention provides a method for synthesising and deconvoluting a combinatorial library comprising the steps of :

(a) apportioning in a stochastic manner among a plurality of reaction vessels a plurality of beads on which a plurality of different compounds can be synthesised, wherein said plurality of beads comprises a population of detectably distinct beads each having a code, such as spatially immobilised particles or vacuoles, which distinctively identifies a respective bead before, during and after said synthesis from other beads,
(b) determining and recording the codes, preferably in the form of the spatial position of the immobilised particles or vacuoles, of said plurality of beads in order to track the movement of individual detectably distinct beads into particular reaction vessels of said plurality of reaction vessels, wherein said codes are determined prior to step (d);
(c) reacting the beads in each reaction vessel with a building block;
(d) pooling the beads from each reaction vessel;
(e) apportioning the beads in a stochastic manner among the plurality of reaction vessels;
(f) reacting the beads in each reaction vessel with another building block;
(g) recording the codes of said plurality of beads in order to track the movement of individual detectably distinct beads into particular reaction vessels of said plurality of reaction vessels, wherein said codes are recorded after step (e) and/or step (f);
(h) pooling the beads from each reaction vessel;
(i) iterating steps (e) through (h) as required in order to create a combinatorial compound library wherein member compounds of the library are associated with the detectably distinct beads and wherein codes of the detectably distinct beads are

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<u>deconvolutable using tracking data provided by said recordal steps to identify the</u>
<u>sequence of reactions experienced by the said detectably distinct beads.</u>

The identification steps (step (c) and (d)) may be effected by use of any suitable
method or apparatus for analysing the spatially immobilised markers of a bead.

Preferably, these steps are effected by flow cytometry, which typically detects opti-
cal parameters. For example, a flow cytometer may be used to determine forward
scatter (which is a measure of size of a bead), side scatter (which is sensitive to
refractive index and size of a particle (seen Shapiro 1995,"Practicalflow cytometry",
3d ed. Brisbane, Wiley-Liss)), and fluorescent emission.

Any suitable algorithm may be employed to track and/or sort individual detectably
unique beads. Preferably, a real-time algorithm is employed.

Suitably, the step of sorting (step (e)) is characterised in that the population of de-
tectably distinct beads constitutes at least about 50%, preferably at least about 70%,
more preferably at least about 90%, and more preferably at least about 95% of the
plurality of beads resulting from step (e).

From the foregoing, a population of detectably unique beads can be generated from
a raw population of beads using e.g. specialised flow cytometric techniques. The
population of detectably unique beads is thereby "pre-encoded" and can be used for
combinatorial synthesis.

<u>Building block reactions</u>

The beads of the invention are applicable to any type of chemical reaction that can
be carried out on a solid support. Such chemical reaction includes, for example:
1. 2 + 2 cycloadditions including trapping of butadiene;
2. [2 + 3] cycloadditions including synthesis of isoxazolines, furans and
modified peptides;
3. acetal formation including immobilization of diols, aldehydes and ketones;
4. aldol condensation including derivatization of aldehydes, synthesis of
propanediols;

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5. benzoin condensation including derivatization of aldehydes;

6. cyclocondensations including benzodiazepines and hydantoins, thiazolidines, -turn mimetics, porphyrins, phthalocyanines;

7. Dieckmann cyclization including cyclization of diesters;

8. Diels-Alder reaction including derivitisation of acrylic acid ;

9. Electrophilic addition including addition of alcohols to alkenes;

10. Grignard reaction including derivatisation of aldehydes;

11. Heck reaction including synthesis of disubstituted alkenes;

12. Henry reaction including synthesis of nitrile oxides in situ (see 2 + 3 cycloaddition);

13. catalytic hydrogenation including synthesis of pheromones and peptides (hydrogenation of alkenes);

14. Michael reaction including synthesis of sulfanyl ketones, bicyclo] 2.2.2] octanes;

15. Mitsunobu reaction including synthesis of aryl ethers, peptidyl phosphonates andthioethers;

16. nucleophilic aromatic substitutions including synthesis of quinolones;

17. oxidation including synthesis of aldehydes and ketones;

18. Pausen-Khand cycloaddition including cyclization of norbornadiene with pentynol;

19. photochemical cyclisation including synthesis of helicenes;

20. reactions with organo-metallic compounds including derivitisation of aldehydes and acyl chlorides;

21. reduction with complex hydrides and Sn compounds including reduction of carbonyl, carboxylic acids, esters and nitro groups;

22. Soai reaction including reduction of carboxyl groups;

23. Stille reactions including synthesis of biphenyl derivatives;

24. Stork reaction including synthesis of substituted cyclohexanones;

25. reductive amination including synthesis of quinolones;

26. Suzuki reaction including synthesis of phenylacetic acid derivatives; and

27. Wittig, Wittig-Horner reaction including reactions of aldehydes; pheromones and sulfanyl ketones.

Reference may also be made to Patel et al., (April 1996, DDT 1 (4): 134-144) who describe the manufacture or synthesis of N-substituted glycines, polycarbarnates,

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mercaptoacylprolines, diketopiperazines, HIV protease inhibitors, 1-3 diols, hydrox-ystilbenes, B-lactams, 1,4-benzodiazepine-2-5-diones, dihydropyridines and dihy-dropyrimidines.

5   Reference may also be made to synthesis of polyketides as discussed, for example, in Rohr (1995, Angew. Int. Ed. Engl. 34: 881-884).

Chemical or enzymatic synthesis of the compound libraries of the present invention takes place on beads. Thus, those of skill in the art will appreciate that the materials
10   used to construct the beads are limited primarily by their capacity for derivitisation to attach any of a number of chemically reactive groups and compatibility with the chemistry of compound synthesis. Except as otherwise noted, the chemically reac-tive groups with which such beads may be derivatised are those commonly used for solid state synthesis of the respective compound and thus will be well known to
15   those skilled in the art. For example, these bead materials may be derivatised to contain functionalities or linkers including-NH2, -NHNH2, -ONH2, -COOH, -SH, -SeH, -SO3H, -GeH, or -SiR2H groups.

Linkers for use with the beads may be selected from base stable anchor groups as
20   described in Table 2 of Fruchtel et al. (1996, supra, the entire disclosure of which is incorporated herein by reference) or acid stable anchor groups as described in Ta-ble 3 of Fruchtel et al. (1996, supra). Suitable linkers are also described in W093/06121, which is incorporated herein by reference.

25   In the area of peptide synthesis, anchors developed for peptide chemistry are stable to either bases or weak acids, but for the most part, they are suitable only for the immobilisation of carboxylic acids. However, for the reversible attachment of special functional groups, known anchors have to be derivatised and optimised or, when necessary, completely new anchors must be developed. For example, an anchor
30   group for immobilisation of alcohols is (6 hydroxymethyl)-3,4 dihydro-2H-pyran, whereby the sodium salt is covalently bonded to chloromethylated Merrifieldz resin by a nucleophilic substitution reaction. The alcohol is coupled to the support by elec-trophilic addition in the presence of pyridinium toluene-4 sulphonate (PPTS) in di-chloromethane. The resulting tetrahydropyranyl ether is stable to base but can be
35   cleaved by transetherification with 95% trifluoroacetic acid. Benzyl halides may be

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coupled to a photolabile sulfanyl-substituted phenyl ketone anchor.

It will also be appreciated that compounds prepared with the beads and/or process of the present invention may be screened for an activity of interest by methods well known in the art. For example, such screening can be effected by specialised flow cytometry invented from standard techniques such as described e.g. by Needels et al. (1993, Proc. Natl. Acad. Sci. USA 90: 1070010704, incorporated herein by reference), Dower et al. (supra), and Kaye and Tracey (WO 97/15390, incorporated herein by reference).

Synthesis of a combinatorial compound library

A combinatorial library in accordance with the present invention is a collection of multiple species of chemical compounds comprised of smaller subunits or monomers. Combinatorial libraries come in a variety of sizes, ranging from a few hundred to many hundreds of thousand different species of chemical compounds. There are also a variety of library types, including oligomeric and polymeric libraries comprised of compounds such as peptides, carbohydrates, oligonucleotides, and small organic molecules, etc. Such libraries have a variety of uses, such as immobilization and chromatographic separation of chemical compounds, as well as uses for identifying and characterizing ligands capable of binding an acceptor molecule or mediating a biological activity of interest.

The library compounds may comprise any type of molecule of any type of subunits or monomers, including small molecules and polymers wherein the monomers are chemically connected by any sort of chemical bond such as covalent, ionic, coordination, chelation bonding, etc., which those skilled in the art will recognize can be synthesized on a solid-phase support

The term polymer as used herein includes those compounds conventionally called heteropolymers, i.e., arbitrarily large molecules composed of varying monomers, wherein the monomers are linked by means of a repeating chemical bond or structure. The polymers of the invention of this types are composed of at least two subunits or monomers that can include any bi-functional organic or herteronuclear molecule including, but not limited to amino acids, amino hydroxyls, amino isocy-

anates, diamines, hydroxycarboxylic acids, oxycarbonylcarboxylic acids, aminoalde-
hydes, nitroamines, thioalkyls, and haloalkyls.

In the disclosure of the present invention, the terms "monomer," "subunits" and
"building blocks" will be used interchangeably to mean any type of chemical building
block of molecule that may be formed upon a solid-phase support. The libraries are
not limited to libraries of polymers, but is also directed to libraries of scaffolded small
molecules.

Various techniques for synthesizing libraries of compounds on solid-phase supports
are known in the art. Solid-phase supports are typically polymeric objects with sur-
faces that are functionalized to bind with subunits or monomers to form the com-
pounds of the library. Synthesis of one library typically involves a large number of
solid-phase supports.

To make a combinatorial library, solid-phase supports are reacted with a one or
more subunits of the compounds and with one or more numbers of reagents in a
carefully controlled, predetermined sequence of chemical reactions. In other words,
the library subunits are "grown" on the solid-phase supports. The larger the library,
the greater the number of reactions required, complicating the task of keeping track
of the chemical composition of the multiple species of compounds that make up the
library. Thus, it is important to have methods and apparatuses which facilitate the
efficient production of large numbers of chemical compounds, yet allow convenient
tracking of the compounds over a number of reaction steps necessary to make the
compounds.

Combinatorial libraries represent an important tool for the identification of e.g. small
organic molecules that affect specific biological functions. Due to the interaction of
the small molecules with particular biological targets and their ability to affect spe-
cific biological functions, they may also serve as candidates for the development of
therapeutics. Accordingly, small molecules can be useful as drug leads eventually
resulting in the development of therapeutic agents.

Because it is difficult to predict which small molecules will interact with a biological
target. intense efforts have been directed towards the generation of large numbers,

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or "libraries", of small organic compounds. These libraries can then be linked to sensitive screens to identify the active molecules.

A number of libraries have been designed to mimic one or more features of natural
5   peptides. Such peptidomimetic libraries include phthalimido libraries (WO 97/22594), thiophene libraries (WO 97/40034), benzodiazopene libraries (U.S. Pat. No. 5,288,514), libraries formed by the sequential reaction of dienes (WO 96/03424), thiazolidinone libraries, libraries of metathiazanones and their derivatives (U.S. Pat. No. 5,549,974), and azatide libraries (WO 97/35199) (for review of pepti-
10   domimetic technologies, see Gante, J., Angew. Chem. Int. Ed. Engl. 1994, 33, 1699-1720 and references cited therein).

The present invention also resides in a method of synthesising and deconvoluting a combinatorial library as described herein above. The codes of the plurality of beads
15   are determined preferably before the first reaction step, although codes may be determined at any time before the first pooling step (step (d), cf. method steps cited above).

Preferably, every time the plurality of beads is apportioned into reaction vessels,
20   each one of the vessels is analysed to determine which of the detectably distinct beads are in each reaction vessel. A database of all the beads (or corresponding gridspaces, supra) can thus be updated to show the synthetic history of the compound synthesised on each bead.

25   During a reaction step, the beads in each reaction vessel are reacted with a building block required to assemble a particular compound. Assembly of compounds from many types of building blocks requires use of the appropriate coupling chemistry for a given set of building blocks. Any set of building blocks that can be attached to one another in a step-by-step fashion can serve as the building block set. The attach-
30   ment may be mediated by chemical, enzymatic, or other means, or by a combination of these. The resulting compounds can be linear, cyclic, branched, or assume various other conformations as will be apparent to those skilled in the art. For example, techniques for solid state synthesis of polypeptides are described, for example, in Merrifield (1963, J. Amer. Chem. Soc. 35: 2149-2156). Peptide coupling chemistry is
35   also described in"The Peptides", Vol. 1, (eds. Gross, E., and J. Meienhofer),

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Academic Press, Orlando (1979), which is incorporated herein by reference.

To synthesise the compounds, a large number of the beads are apportioned among a number of reaction vessels. In each reaction, a different building block is coupled to the growing oligomer chain. The building blocks may be of any type that can be appropriately activated for chemical coupling, or any type that will be accepted for enzymatic coupling.

Because the reactions may be contained in separate reaction vessels, even building blocks with different coupling chemistries can be used to assemble the oligomeric compounds (see, The Peptides, op. cit). The coupling time for some of the building block sets may be long. For this reason the preferred arrangement is one in which the building block reactions are carried out in parallel. After each coupling step, the beads on which are synthesised the oligomers or compounds of the library are pooled and mixed prior to re-allocation to the individual vessels for the next coupling step. This shuffling process produces beads with many oligomer sequence combinations. If each synthesis step has high coupling efficiency, substantially all the oligomers on a single bead will have the same sequence. That sequence is determined by the synthesis pathway (building blockreactions and the order of reactions experienced by the beads) for any given bead. The maximum length of the oligomers may be about 50, preferably from 3 to 8 building blocks in length, and in some cases a length of 10 to 20 residues is preferred. Protective groups known to those skilled in the art may be used to prevent spurious coupling (see, The Peptides, Vol. 3, (eds. Gross, E., and J. Meienhofer), Academic Press, Orlando (1981), which is incorporated herein by reference).

With enough beads and efficient coupling it is possible to generate complete sets of certain oligomers, if desired. The appropriate size of the beads depends on (1) the number of oligomer synthesis sites desired; (2) the number of different compounds to be synthesised (and the number of beads bearing each oligomer that are needed for screening); (3) the effect of the size of the beads on the specific screening strategies e. g. fluorescence-activated cell sorters (FACS) to be used; and (4) the resolution of the encoding/detection methods employed.

<u>Kit of parts</u>

The invention in a still further aspect resides in a kit comprising:

5   a combinatorial compound library including a plurality of different
         compounds wherein each compound is attached to at least one of a plurality of
         beads, which includes a population of detectably distinct beads each having a dis-
         tinctive code, which distinctively identifies a respective bead before, during and after
         synthesis of a corresponding compound from other beads; and

10   tracking data on each distinctive code to identify the sequence of reactions
         experienced by a respective detectably distinct bead.

The invention in a yet further aspect resides in a kit comprising:

15   a composition of spatially encoded polymer matrices according to the present inven-
         tion comprising a plurality of spatially immobilised particles; and

         an encoded bead reader device according to the invention for identifying and re-
20   cording individual encoded beaded polymer matrices, wherein the device is option-
         ally linked to a computer running a program for calculating distance matrices for
         individual, spatially encoded, beaded polymer matrices.

Examples

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General Methods.

Reagents were obtained from Fluka and used without any purification. All solvents
used were of HPLC grade kept over molecular sieves. Oregon green was obtained
30   from Molecular Probes. The 28-53 μm beads were prepared in a specially designed
         high-speed stirred polymerisation steel reactor and 5-28 μm beads were prepared
         by using a high-speed dispersion reactor. The encoded macro beads were prepared
         in a 250 ml baffled glass reactor equipped with a dispersion stirrer. The fluorescence
         images were obtained with a microscope and a digital camera. Broad band excita-

tion in the near UV range was provided by a mercury lamp. The images of the encoded beads were recorded in water.

Example 1: Preparation of encoded $(NH_2)_2PEG_{1900}$-Acrylamide copolymer beads.

Labelled microbeads encoded $(Acr)_{1.4}$ $(NH_2)_2PEG_{1900}$-acrylamide were prepared by inverse suspension polymerisation method. In order to prepare the beads having a size 500 μm, a lower wt% (1.4%) of sorbitan monolaurate with the macromonomer was used as the suspension stabiliser. The n-heptane was used as the suspension medium and was degassed with argon for 1 h before the addition of monomers. In a typical synthesis procedure, a solution of $(Acr)_{1.4}$ $(NH_2)_2PEG_{1900}$ (7.3 g, 3.54 mmol) in water (21 mL) was degassed with argon for 30 min. Acrylamide (0.36 g, 5 mmol) and the labelled micro beads (20 mg) in water (0.5 mL) were added to the degassed solution and the purging of argon was continued for 5 min. A solution of sorbitan monolaurate (0.1 mL) in DMF (1 mL) and the free radical initiator ammonium persulfate (300 mg) in water (2 mL) were added to the monomer mixture. The reaction mixture was then rapidly added to the suspension medium and stirred at 600 rpm at 70 °C. After one min, TEMED (1.5 mL) was added to the reactor. The reaction was allowed to continue for 3h, the encoded beads formed were filtered through the sieves and the 500 μm fraction was collected. The beads were washed thoroughly with ethanol (10×), water (10×), ethanol (10×) and dried under high vacuum.

Example 2: Preparation of microbeads for encoding

Synthesis of partially acryloylated $(NH_2)_2PEG_{500}$ and $(NH_2)_2PEG_{1900}$
Acryloyl chloride (1.267 mL, 14 mmol) in DCM (12 mL) was added dropwise to a solution of $(NH_2)_2PEG_{500}$ (6.3 g, 10 mmol) in DCM (18 mL) at 0 °C with stirring. The reaction mixture was kept for 1 h at 20 °C. The DCM was evaporated and drying in vacuo at 20 °C yielded the 70% acyloylated $(NH_2)_2PEG_{500}$ as colourless thick oil. The partially acryloylated $(NH_2)_2PEG_{1900}$ was prepared by following the same procedure with $(NH_2)_2PEG_{1900}$ (20 g, 10 mmol) in DCM (12 mL) and acryloyl chloride (1.267 mL, 14 mmol) in DCM (18 mL).

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Synthesis of $(Acr)_{1.4}$ $(NH_2)_2PEG_{500}$-DMA micro beads (28-53 µm) :

A: using high speed stirred reactor:

Beads of $(Acr)_{1.4}$ $(NH_2)_2PEG_{500}$-DMA (28-53 µm) were prepared by the inverse suspension polymerisation of aqueous solutions of monomers in n-heptane:CCl_4 mix-
ture(6:4, v/v, 240 mL). Sorbitan monolaurate was used by 8 wt % of the macro-monomer for the stabilisation of the suspension. Argon was bubbled to the n-hepane-CCl_4 mixture for 1 h before the addition of monomers. In a typical synthesis procedure, a solution of $(Acr)_{1.4}$ $(NH_2)_2PEG_{500}$ (6.6 g, 10 mmol) in water (21 mL) was degassed with argon for 30 min. DMA (343µL, 3.32 mmol) was added to the de-
gassed solution and the purging of argon was continued for 5 min. A solution of sorbitan monolaurate (0.5 mL) in DMF (2 mL) and the free radical initiator ammonium persulfate (200 mg) in distilled water (1 mL) were added to the monomer mixture. The reaction mixture was then rapidly added to the suspension medium in the polymerisation reactor stirred at 2500 rpm at 70 °C. After one min, TEMED (1 mL) was
added to the reactor. The reaction was allowed to continue for 3h, the microbeads formed were filtered through the sieves and the 28-53 µm fractions were collected. The microbeads were washed thoroughly with ethanol (10×), water (10×), ethanol (10×) and dried under high vacuum.

B: using dispersing instrument

Microbeads of $(Acr)_{1.4}$ $(NH_2)_2PEG_{500}$ (5-28 µm) were prepared by the inverse suspension polymerisation of aqueous solutions of monomer in n-heptane (100 mL). Sorbitan monolaurate was used by 25 wt % of the macromonomer for the stabilisation of the suspension. Argon was bubbled to the n-hepane for 1 h before the addi-
tion of monomer. A solution of $(Acr)_{1.4}$ $(NH_2)_2PEG_{500}$ (2 g, 3.03 mmol) in water (6 mL) was degassed with argon for 30 min. A solution of sorbitan monolaurate (0.5 mL) in DMF (1 mL) and the free radical initiator ammonium persulfate (200 mg) in distilled water (1 mL) were added to the monomer solution. The reaction mixture was then rapidly added to the suspension medium in a reactor equipped with a high-
speed dispersing instrument stirred at 9000 rpm at 70 °C. After one min, TEMED (1 mL) was added to the reactor. The reaction was allowed to continue for 3h, the microbeads formed were filtered through the sieves and the 5-28 µm fractions were collected. The microbeads were washed thoroughly with ethanol (10×), water (10×), ethanol (10×) and dried under high vacuum.

Example 3: Labelling of encoding particles.

Labelling of $(Acr)_{1.4}$ $(NH_2)_2PEG_{500}$-DMA (28-53 µm) micro beads with Oregon Green 514 dye: The microbeads (0.2 g, 0.8 mmol/g) were kept in DMF/water (5 mL) for 1h. The Oregon Green™514 carboxylic acid, succinimidyl ester (0.147 g, 0.24 mmol) in DMF (200 µL) was added to the swollen microbeads and the reaction mixture was kept at room temperature. After 1 h, the beads were filtered through a 0.45 micron filter and washed with DMF (10×) and water (10×).

Labelling of $(Acr)_{1.4}$ $(NH_2)_2PEG_{500}$ (5-28 µm) micro beads with Oregon Green 514 dye: The microbeads (0.2 g, 1 mmol/g) were kept in DMF/water (5 mL) for 1h. The Oregon Green™514 carboxylic acid, succinimidyl ester (0.184 g, 0.3 mmol) in DMF (200 µL) was added to the swollen microbeads and the reaction mixture was kept at room temperature. After 1 h, the beads were filtered through a 0.45 micron filter and washed with DMF (10×) and water (10×).

Labelling of $(Acr)_{1.4}$ $(NH_2)_2PEG_{500}$-DMA (28-53 µm) micro beads with 1-Cyano benz[f]isoindole: To a stirred suspension of 2,3-naphthalene dicarboxaldehyde (29.44 mg, 0.16 mmol) in MeOH (2 mL) was added NaCN (8 mg, 0.16 mmol) at room temperature. To the reaction mixture, the resin (0.2 g, 0.16 mmol) was added and kept at room temperature. After 30 min, the resin was filtered and washed with MeOH (10×), DMF (10×) and water (10×).

Example 4: Presorting beads according to integrated fluorescence.

A custom made Compas Beads (Union Biometrica) beadsorter with laser exitation at 488 nm and detection of fluorescence at 514 nm was used to pool the beads synthesized according to the number if small particles. The integrated fluorescence of the small particles was recorded with the selection (sorting) window preset to collect those beads having 3-4 small particles at a sorting rate of 30 beads / s at a flowrate of 1000 mm / s of Compas sheat fluid corresponding to sorting of 900.000 beads in a working day. The collected beads were resorted to yield approximately ~20% containing 3 – 4 particles / bead. The quality of the collected pool was veryfied us-inga fluorescence microscope.

Example 5: Synthesis of 400 dipeptides with bead portioning and bead identification.

The peptide library was prepared in a 20-well multiple column peptide synthesiser. Approximately 50 beads were taken in a glass plate and the image of these beads were recorded in three shuffled states and then added to one of the wells in the synthesiser. The beads were taken in 20 wells of the synthesiser accordingly. The resin was washed with DMF and the N_α-Fmoc-protected OPfp ester of the amino acid (10 mg) was added to each well of the synthesis block. The reaction mixture was kept at room temperature for 3 h and washed with DMF (6×). The beads were removed from the block, combined together and the Fmoc-protection was removed by 20% piperidine in DMF (3mL, 20 min). The resin was washed with DMF (10×), split in to 20 portions and added to each well of the block after recording its image in three shuffled states. After the incorporation of the second amino acid, the beads were transferred to a syringe. The Fmoc protection was removed by 20% piperidine in DMF (3 mL, 20 min) and the resin was washed with DMF (10×). The side chain protection of the peptide was removed by treating with 50% TFA in DCM (3 mL, 30 min), and the resin was washed with DCM (10×), DMF (10×) and water (10×).

Example 6: Selection and structure determination on a fraction of an encoded library by visual decoding.

Twenty beads were randomly selected from the peptidyl resin and record the images separately in water. The sequence of the dipeptide on each bead was decoded by visual comparison of final image of the bead with pre-recorded images of the beads.

Example 7: Confirmation of structure by solid phase Edman sequencing.

Single beads from the dipeptide library were placed on a filter and subjected to Edman sequencing on a 477 A Protein Sequencer (Applied Biosystems) to provide the dipeptide structure in two standard cycles.

Example 8: Capturing 3 orthogonal 2D-projections of a bead

A triangular hole was carved in a 1 cm plate of POM. The hole was symmetrical with sides angled at 54.3 ° and a length of the side of the lower triangle of 5 mm. The plate was mounted horisontally and a microscope was mounted at an angle of 35.7 ° underneath so it was perpendicular to the surface of a quartz flowcell mounted in the triangular hole of the POM holder. The corner of the quartz cell could thus be projected onto the CCD of the microscope from all three orthogonal sides, simple by carefull rotation of the quartz cell. The beads recorded were fixed on the quartz cell wall simply by adhesion to the walls of the corner and was submerged in the appropriate solvent. Three orthogonal pictures were recorded under identical conditions and the coordinates relative to one of the particles were generated.

Example 9: Determining the centre of a particle in a 2-D projection.

The three 2-D pixel-based projections obtained from CCD cameras are treated by the following algorithm. For each alternate pixel in each alternate line of the image it was tested whether a pixel was bright or dark by testing the blue rgb value. Testing was continued from the one before the bright pixel in single pixel steps until at least two dark pixels were detected. Then the center of the range of bright pixels were determined. From this point the height and the center of the bright spot was determined. The center and the region occupied by the bead was recorded. The search for spots was continued, but ommitting bright pixels within areas already occupied. The centers of bright spots with an area above a small treshold were used as coordinates for the particles.

Example 10: Confocal determination of spatial positions of particles in a bead

In a stopped flow system individual beads are positioned in a confocal scanning system as in a commercial scanning microscope. The positioning system is based on small IR lasers detecting changes in refractive index or absorption. If the particles are fluorescent, the bead is illuminated with monochromatic light corresponding to excitation wavelength of the fluorescent dye. If it is simple coloured particles the bead is illuminated with white light.

The bead is scanned in consecutive 2-D layers as depicted in figure 3. The resolution (pixel dimension) in the layers as well as the distance between layers is selected to be smaller than the average particle diameter. Based on the consecutive scans a 3-D matrix of particle positions are formed. The dataset is reduced calculat-

5   ing inter-particle distances or vectors, and the remaining information is discarded.

Example 11: Recording of coordinates of particles in a moving bead by two alternating scanning lasers

10   In a pulsation free constant flow system individual beads are passed through a scanning system with two orthogonal laser scanning systems as depicted in figure 4. The two 1-D laser scanners are both orthogonal to the flow direction, and allow a full 3-D scanning of the passing beads, which are moving with constant velocity. In fluorescence mode the lasers will emit light at an excitation wavelength for the fluores-

15   cent dye in the particles in the beads.

A fast response emission light detector records the time-resolved emission signal, which in conjunction with the flow speed and the scan parameters are used to construct a full 3-D matrix of particle positions. The dataset is reduced calculating inter-

20   particle distances or vectors, and the remaining information is discarded.

Example 12: Recording of coordinates of particles in a moving bead by rotational scan

25   In a pulsation free constant flow system individual beads is passed a scanning system applying a rotational scan focussed on the bead via a parabolic mirror as depicted in figure 4.

The two 1-D laser scanners are both orthogonal to the flow direction, and allow a full

30   3-D scanning of the passing beads, which are moving with constant velocity. In fluorescence mode the laser emits light at an excitation wavelength for the fluorescent dye in the particles in the beads. The circle scan onto the parabolic mirror is passing alternating 60 degree segments of full and blocked transmission to give three curved scan lines for each rotation.

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A fast response emission light detector records the time-resolved emission signal, which in conjunction with the flow speed and the scan parameters are used to construct a 3-D matrix of particle positions. The dataset is reduced calculating inter-particle distances or vectors, and the remaining information is discarded.

Example 13: Determination of distance matrix from particle coordinates.

The coordinates of the particles were determined according to example 8 above. Coordinates were measured in pixel units e.g for picture set b1a-b1c coordinates (0, 0, 0); (22, 110, 84); (-94, 168, 153) were measured. From these coordinates the unique set of distance parameters (140, 245, 146) (length of inter particle vectors) were calculated according to the formula presented in Figure 1. The average error on determination of coordinates was approximately 2 % corresponding to the resolution of the method.

Example 14: Robustness of the method for identification.

In order to obtain a quantitative measure of how well individual beads can be distinguished by inserting small fluorescent beads to encode them, a Monte Carlo simulation was performed.

To this end, envisage a spherical bead as being composed of small cubic volume elements (voxels) of unit vertex length. The actual voxel size corresponds to the accuracy of determining the position of the fluorescent marker within the bead. Then the voxel centers form a grid of potential encoding points within the spherical bead, out of which in the simplest cases m=3 or m=4 are randomly selected. Generically, these points of encoding can be regarded as corners of a triangle with n=3 vertices or of a tetrahedron (n=6 vertices). The vertex lengths correspond to the distances between the encoding points. When ordered by magnitude, the set of vertex lengths is invariant under global rotation and translation of the large bead. (Moreover, it is in fact invariant under any action of a symmetry point group in three-dimensional space including inversion at the origin and mirroring at a plane thus discarding potential discrimination by chirality in the case of tetrahedra.)

If the set of vertex lengths is ordered in descending magnitude, i.e. $v_1 \geq v_2 \geq ... \geq v_n \geq 0$ with $n = 3$ (6), then the encoding vector

$\mathbf{v} = (v_1, v_2, ..., v_n)$ can be identified with a point in the upper half of the three (or six-) dimensional positive real space J^3_+ (J^6_+), respectively.

Let $r \geq 0$ denote the finite resolution of a CCD camera. Then two beads j and k cannot be discerned if their encoding vectors v_j and v_k correspond to points less than r apart from one another, i.e. if the separation of encoding $\cap v = \tau v_j - v_k \tau$ falls below the camera resolution $\cap v$ P r.

The corresponding Monte Carlo simulation gives the following results for a bead of 500 units diameter: If the bead is marked with 3 fluorescent points, then 5 in 10'000 beads give rise to an encoding separation $\cap v$ P 6 units. If 4 encoding points are used as shown in Fig. 8, then only 2 in 10'000 beads show separations $\cap v$ P 12 units, and in none out of 50 million pairs simulated the separation is $\cap v$ P 3 units. For a bead diameter of 5000 units the resolution greatly improves: Encoding by 3 points leads to 5 beads in 100'000 whose separations of encoding are $\cap v$ P 60 units. With 4 points one only has 1 in 100'000 beads with $\cap v$ P 120 units separation.

In conclusion, by inserting four encoding particles into a standard bead of 500 μm diameter, within which the centers of the fluorescent particles can be determined with a typical accuracy of 1 μm, provides ample space for encoding many millions of individual beads. The probability that an active hit will not be uniquely identified is very small according to the present simulations.

Example 15. Microbead synthesis

In order to optimize the synthesis of the microbeads, 4 experiments were made. In these experiments the stirring speed and the amount of surfactant were changed, see Table 2.

Table 2: Conditions for optimization of microbeads

Sample	Sorbitain monolaurate (g / %)	Acr-JEF$_{600}$ (g)	Dimethyl acrylamide (ml)	H$_2$O (g)	Vazo 44 (mg)	TMEDA (ml)	Stirring rate (rpm)
JHT466	1.65/25	6.6	0.34	22	283	1 ml	4000

JHT471	1.65/25	6.6	0.34	22	283	-	2000
JHT472	0.825/12.5	6.7	0.34	22	283	1 ml	2000
JHT473	0.825/12.5	6.6	0.34	22	283	1 ml	4000

All polymerizations were made in 270 ml Isopar M, using a polymerization temperature of 30°C (beginning) to 50°C (end) and a reaction time of 4 h.

5   For a typical synthesis, 240 ml Isopar M was transferred to a baffled steel reactor and heated to the polymerization temperature. The Isopar M was purged with argon for 30 minutes. In a round-bottomed flask 6.6 g AcrJeffamine-600 (AcrJEF$_{600}$), 0.825 g sorbitane monolaurate, 22 g distilled water and 0.34 ml dimethyl acrylamide were mixed and purged with argon for 30 minutes. After 30 minutes 0.283 g of Vazo

10   44 was added and the polymerization solution was purged with argon for another 5 minutes before it was transferred to the reactor. The polymerization solution was suspended in the Isopar M using a stirring speed of 2000 rpm. After 1 minute 1 ml of N,N,N',N'-tetra methyl ethylene diamine (TMEDA) was added to the polymerizing suspension The polymerization temperature was initially 30°C but it was increased

15   to 50°C in 1 h. The suspension was stirred for another 3 h.

The microbeads were purified by centrifugation using ethanol and dimethyl formamide (DMF) for washing. In a typical purification the suspension from the reactor was transferred to two centrifuge tubes and centrifuged at 4000 rpm for 20 minutes.

20   The supernatant was removed and the beads were suspended in ethanol. The suspension was centrifuged at 4000 rpm for 20 minutes and the supernatant removed. This procedure was repeated until the supernatant was clear. Afterwards, the microbeads were washed 3 times with DMF using the procedure just described.

25   For the use in the encoded beads the micro-beads were labeled   using a flurissen dye. In a typical reaction 1.0 g of microbeads swelled in DMF, 34 mg ATOTA 1 (flurissen dye) and 20 ml of DMF were mixed in a 50 ml round-bottomed flask. The flask was fitted with a condenser and a drying tube. The mixture was reacted for 2 days. The purification of the beads is described in the part describing the purification

30   and fractionation of the microbeads after labelling. Fig. 16 shows pictures of the purified microbeads before fractionation.

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As observed from the pictures in Fig. 16, the microbeads JHT466 and 473 are very small, and cannot be used for encoded beads. The bead size distribution in sample JHT472 is broad and the average size to small. The best result was obtained using the conditions given for JHT471, however, some separation of the beads are still
5   needed.

Example 16. Spatially encoded beads

In order to optimise the preparation of encoded beads, a range of experiments were
10   made. The aim was to improve the distribution and number of microbeads in the encoded beads and to improve the strength of the encoded beads. The conditions for reactions made in this optimization are given in Table 3.

In a typical encoded beads preparation, a baffled steel reactor was charged with 270
15   ml of Isopar M and heated to 70°C. The Isopar was purged with argon for 30 minutes. 16.9 g AcrJeffamine-2000 (AcrJEF$_{2k}$), 0.839 g acrylamide, 0.231 g sorbitain monolaurate, 200 µl microbeads in DMF and 50 g distilled water were mixed in a 250 ml round-bottomed flask. The mixture was purged with argon for 30 minutes before 0.695 g of ammonium persulfate was added. Afterwards, it was purged with
20   argon for another 5 minutes before it was transferred to the reactor. The polymerizing mixture was suspended in the Isopar M using a stirring speed of 500 rpm. After 1 minute 3.4 ml of TMEDA was added. The polymerizing suspension was stirred for 5 h. at 70°C. The polymerization temperature of 70°C was constant over night to ensure high conversion. Afterwards, the Isopar M was removed by filtration and the
25   product was washed with 3*300 ml dichloromethane, 3*300 ml tetrahydrofurane, 3*300 ml methanol and 5*300 ml distilled water. The results from the optimization are given in Table 3. All the polymerizations were made in 270 ml Isopar M, except for JHT475 and JHT483 which were made in 270 ml n-Heptane. Vazo 44 or Ammonium persulfate were used as radical initiators. A fluorescence image of one en-
30   coded bead from sample JHT476 is shown in figure 17. An orthogonal pair of fluorescence images obtained with an encoded bead reader according to the present invention is shown in figure 18.

Table 3: Reaction conditions for encoded beads synthesis.

Sample	AcrJEF$_x$ (g)	Sorbitain monolaurate (g)	Acryl-amide (g)	Particles (μl)	Radical initiator (g)	TMEDA (ml)	Temp (°C)	Stirring (rpm)	H$_2$O (g)
JHT469	16.9 x = 2k	0.231	0.840	50[a]	0.424[b]	3.4	40	400	50
JHT474	16.9 x = 2k	0.231	0.840	100[a]	0.424[b]	3.4	40	300	50
[c] JHT475	16.9 x = 2k	0.231	0.840	80[a]	0.424[b]	3.4	40	300	50
JHT476	16.5 x = 900	0.083	0.834	100[d]	0.762[b]	1.44	40	300	63
JHT477	16.7 x = 900	0.083	0.837	75[d]	0.762[b]	1.44	50	400	63
JHT478	16.5 x=900	0.083	0.838	75[d]	0.762[b]	1.44	50	500	44
JHT479	16.5 x = 600	0.083	0.838	75[d]	0.762[b]	1.44	50	500	63
JHT480	16.5 x = 900	0.083	0.860	200[d]	0.536[e]	1.44	70	500	64
JHT481	16.6 X = 900	0.085	0.834	-	0.377[e]	1.44	70	450	63
JHT482	16.9 x = 2k	0.231	0.844	200[d]	0.695[f]	3.4	70	500	50
[c] JHT483	16.9 x = 2k	0.231	0.839	50[d]	0.695[f]	3.4	70	500	50
JHT484	16.9 x = 2k	0.301	0.839	50[d]	0.695[f]	3.4	70	400	50

a) JHT466

b) Vazo 44, mixing temperature: 0°C

c) N-Heptane was used as continuous phase

d) JHT71 (the small beads was not removed by centrifugation)

e) Ammonium persulfate, mixing temperature: RT'

f) JHT71 (the small beads was removed by centrifugation)

Example 17: Numerical simulations of encoded bead identification

The process of reading and identifying encoded beads code was numerically simu-
lated using the MatLab by the following method:

1. Forming a virtual set of spatially encoded beads in a computer on the
   basis of the following set of spatially encoded bead properties: total
   number of encoded beads 5000, encoded bead diameter distribution 0.7

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– 1.0 micrometers, immobilised particle diameter distribution 5 – 14 micrometers, number of particles per encoded bead distribution 4 – 8, average number of immobilised particles per encoded bead 5, uncertainty involved in the determination of the spatially immobilised particle positions 1 micrometer,

2. Simulating random rotation of all spatially encoded beads,

3. Computing one pair of orthogonal projections of each of the spatially immobilised particles of each spatially encoded bead,

4. Combining the two orthogonal sets of 2D-positions whereby the set of possible 3D-positions is obtained for each spatially encoded bead,

5. Computing the set of distance matrices corresponding to the set of 3D-positions thus determined,

6. Identifying single spatially encoded beads by comparing the full set of distance matrices of single spatially encoded beads against the full set of distance matrices of all spatially encoded beads. The best fit of single distance matrices hereby obtained identifies single spatially encoded beads.

7. Registering the number of not-identified spatially encoded beads,

2. Repeating the sequence 1 to 7 three times with the following values of the average number of immobilised particles per encoded bead: 5, 6, and 7.

3. Repeating the steps 1 to 8 four times with the following values of the uncertainty involved in the determination of the spatially immobilised particle positions: 1, 2, 4, and 8 micrometers.

The result is presented in the two charts in figure 19 in terms of the number of encoded beads with correspondence problem and the number of not-identified spatially encoded beads. It can be seen from the figure that the number of encoded beads with correspondence problem and the number of not identified encoded beads increase with the uncertainty involved in the determination of the spatially immobilised particle positions. It further appears from the figure that the number of encoded beads with correspondence problem increases with increasing average number of immobilised particles per encoded bead, whereas the number of not identified encoded beads does not vary significantly with the average number of immobilised particles per encoded bead.

Example 18: Use of triangles defined by the microbeads for identification of encoded beads

5   A batch of encoded beads was prepared according to the method described in Example 17 with the set of parameters used for the batch termed JHT476. The beads were sorted according to size by sifting, and 60 beads from the 500 – 700 micrometers diameter fraction were dispersed in water and fed to an encoded bead reader device comprising a quart flow cell with a rectangular cross-section, manual sy-

10   ringes for passing a bead dispersion through the flow cell, a 473nm laser for illuminating a central section of the flow cell, two orthogonally aligned cameras equipped with image intensifiers, optical objectives, and band pass fluorescence filters, an image framegrabber for transferring the images from the cameras to a computer, a harddrive for storing the images, an electronic pulse generator for controlling the

15   cameras, the image intensifiers, and the framegrabber.

Each bead was gently passed through the flow cell whereby it was ensured that the bead rolled on the lower horizontal inner wall of the flow cell. The resulting image sequence consist of a series of orthogonal pairs of images of the bead imaged from

20   various angles. For each pair of orthogonal images one distance matrix, D, was derived:

$$D = \begin{bmatrix} d_{11} & d_{12} & ... & d_{1N} \\ d_{21} & d_{22} & ... & d_{2N} \\ \vdots & \vdots & \ddots & \vdots \\ d_{N1} & d_{N2} & ... & d_{NN} \end{bmatrix}$$

where N is the number of microbeads observed in the bead, and d_{jk} is the distance between microbead j and microbead k, where $j = 1, 2, ..., N$ and $k = 1, 2, ..., N$. Thus

25   $d_{jk} = 0$ when $j = k$, and $d_{jk} = d_{kj}$.

Two pairs of orthogonal images from one such image sequence of an encoded bead are shown in figure 20. In the lower pair of images in the figure the bead is rotated about 15° compared to the upper pair of images. The images to the left were ob-

30   tained with a camera looking down on the flow cell from above (y,z-projections). The images to the right were obtained with a camera looking at the flow cell from the

side (x,z-projections). Also shown in the figure is the result of the automatic determination of the microbead positions marked with "+". Only microbeads that are observed at the same z-position in the y,z- and the x,z-projection are considered for the distance matrix determination. The six different distances between the four microbeads considered are given in figure 20 for each pair of orthogonal images to the right of each of the two image pairs. Ideally the distances should be identical, however, deviations in the range 0.2 – 1.4 pixels are observed corresponding to a distance determination reproducibility in the range 0,5 – 3,5 μm.

From the distance matrix of one pair of images all possible triangles defined by the considered microbeads are generated in the computer. Each of the triangles are then stored as a vector, $L_i = [l_{i1}, l_{i2}, l_{i3}]$, where l_{i1}, l_{i2}, and l_{i3} are the respective distances between the three microbeads defining triangle i, where $i = 1, 2, ..., M$, and M is the number of possible triangles for the given number of microbeads. The full set of triangles of each encoded bead are then stored for use in the later identification procedure. This is done for all encoded beads, and from the resulting sets of triangles a look-up table is generated. With the use of this look-up table any one of the encoded beads can be identified at a later stage on the basis of its unique set of triangles as defined by its microbead geometry.

Example 19: Preparing spatially encoded polymer beads

Spatially encoded PEGA-type polymer beads are prepared by inverse phase copolymerisation at 70°C of acrylamide-end-capped polyethyleneglycol and acrylamide in a 1:1 ratio in 2% (w/w) aqueous sorbitan monolaurate in the presence of 0.26% (w/w) di-methylformamide swelled Oregon green (supplied by Molecular probes) dyed microspheres (TentaGel M30202 supplied by RAPP Polymere). After polymerisation the beads were washed with demineralised water and sieved. The resulting 0.7 – 1.0mm diameter fraction is isolated and analysed in the apparatus described in example 18 but with an alternative set of optical objectives, each objective comprising a conventional 10 times magnification microscope objective equipped with a 1.5mm aperture for increasing the focal depth.. About 80% of the encoded beads comprise from 4 to 10 microparticles. Examples of the resulting image pairs are presented in Fig. 21.